Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 7-9, 14-16, 35 and 36 are pending in the application, with claim 7 being the sole independent claim. New claims 35 and 36 are sought to be added. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

I. Support for Amendments

A. In the Specification

Applicants have added to the specification a paragraph captioned "Cross Reference to Related Applications." Support for this paragraph can be found, *inter alia*, in the application data sheet that was included with the originally filed application papers.

B. In the Claims

Support for the amendment to claim 1 and new claim 36 can be found, *inter alia*, in the specification at page 18, line 15, through page 19, line 2; at page 20, line 26, through

page 21, line 2; and at page 21, lines 7-20. Support for new claim 35 can be found, *inter alia*, in the specification at page 13, lines 7-11.

II. Claim Objections

The Examiner has objected to claims 7, 8 and 15 based on the recitation of "Seq. ID No. 1" in these claims. Applicants have amended claims 7, 8 and 15, replacing "Seq. ID No. 1" with "SEQ ID NO:1." Therefore, the Examiner's objection is fully accommodated and should be withdrawn.

III. Claim Rejections Under 35 USC § 112, First Paragraph

A. Written Description

The Examiner has rejected claims 7, 9, 14 and 16 under 35 USC § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that Applicants had possession of the claimed invention at the time the application was filed. *See* Paper No. 12, pages 2-3. Applicants respectfully traverse this rejection.

There are two distinct bases that the Examiner has set forth in support of the written description rejection. First, the Examiner asserted that the specification does not provide sufficient written description for a *DNA molecule* that is at least 40% homologous to SEQ ID NO:1. *See* Paper No. 12, pages 3-4. Second, the Examiner asserted that the specification does not provide sufficient written description for any *transgenic animal* comprising a DNA molecule that is at least 40% homologous to SEQ ID NO:1 and its corresponding phenotype.

See Paper No. 12, pages 4-6. Both of these bases for the rejection are addressed in turn below.

1. A DNA Molecule Which is At Least 40% Homologous to SEQ ID NO:1

The Examiner acknowledged that the specification provides sufficient description of SEQ ID NO:1; however, according to the Examiner, "the as-filed specification does not provide an adequate written description of a representative number of species of nucleotides sequences comprising a DNA molecule with at least 40% homology with SEQ ID NO:1." *See* Paper No. 12, page 3. Although Applicants respectfully disagree with this assessment, Applicants, for purposes of expediting prosecution, have amended claim 7. Claim 7, as currently presented, is directed to a transgenic non-human animal, all of whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, wherein said DNA molecule is expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when expressed in neuronal cells.

Applicants submit that the DNA molecules that are used with and included within the presently claimed invention are described in the specification in sufficient detail such that one skilled in the art would reasonably conclude that Applicants had possession of the claimed invention as of the effective filing date. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991).

In support of the first basis of the written description rejection, the Examiner noted that "the description of the primary sequence of amino acid residues in which the positions

of the amino acid residues are particularly arranged is essential for the biological function of the protein encoded by the sequence." *See* Paper No. 12, page 3. Although the primary sequence of a protein is important for its biological function, the written description requirement may nonetheless be satisfied for a genus of DNA molecules that are at least 90% homologous to a disclosed nucleotide sequence. This proposition is specifically supported by the USPTO's Synopsis of Application of Written Description Guidelines (hereinafter "Written Description Synopsis").

Example 14[1] of the Written Description Synopsis involves an analysis of the following claim: "A protein having SEQ ID NO:3 and variants thereof that are at least 95% identical to SEQ ID NO:3 and catalyze the reaction of A→B." The specification supporting this claim provides the following information:

> The specification exemplifies a protein isolated from liver that catalyzes the reaction of A→B. The isolated protein was sequenced and was determined to have the sequence as set forth in SEQ ID NO:3. The specification also contemplates but does not exemplify variants of the protein wherein the variant can have any or all of the following: substitutions, deletions, insertions and additions. The specification indicates that procedures for making proteins with substitutions, deletions, insertions and additions is routine in the art and provides an assay for detecting the catalytic activity of the protein.

---

[1]Applicants note that Example 14 of the Written Description Synopsis involves an analysis of a claim directed to a *protein molecule*, whereas Applicants' claims are directed to *transgenic animals* and *screening methods* using transgenic animals. Nevertheless, the present claims include or involve the use of the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto. In addition, the Examiner's first basis for the written description rejection is that "the as-filed specification does not provide an adequate written description of a representative number of species of nucleotides sequences comprising a DNA molecule with at least 40% homology with SEQ ID NO:1." Thus, the guidance provided by Example 14 of the Written Description Synopsis is directly relevant to the issue of whether sufficient written description is provided for Applicants' claims.

Written Description Synopsis, Example 14.

The Written Description Synopsis, Example 14, concludes that the disclosure meets the requirements of 35 USC § 112, first paragraph, in part because "procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity are conventional in the art." *See id.* Moreover, it is noted that:

> [t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO:3 which are capable of the specified activity. One of skill in the art would conclude that applicant was in possession of the necessary common attributes possessed by the members of the genus.

Written Description Synopsis, Example 14.

The situation presented in Example 14 of the Written Description Synopsis closely parallels the circumstances surrounding the DNA molecules that are used with, or are found within, Applicants' present claims, and the written description provided therefor. As such, Applicants submit that the guidance and instructions provided by the USPTO for analyzing a claim for compliance with the written description requirement strongly supports Applicants' assertion that the written description requirement of § 112, first paragraph, is satisfied for Applicants' claims.

First, in Example 14, it is stated that "all variants [encompassed by the claim] must possess the specified catalytic activity and must have at least 95% identity to the SEQ ID NO:3." Similarly, all of the species of DNA construct used with, or found within, Applicants' currently presented claims must have at least 90% homology to SEQ ID NO:1

and must code for a protein having an activity of AD7c-NTP when expressed in neuronal cells.

Second, it is noted in Example 14 that "[t]here is a single species disclosed, that species being SEQ ID NO:3;" and that "[t]here is actual reduction to practice of the single disclosed species." Likewise, Applicants have disclosed SEQ ID NO:1 in the specification and have shown actual reduction to practice of SEQ ID NO:1. *See* specification at page 33, line 9, through page 35, line 28 (describing the isolation of the AD7c-NTP cDNA and the characteristics of the molecule); *see also* Fig. 1.

Third, according to Example 14, "procedures for making variants of SEQ ID NO:3 which have 95% identity to SEQ ID NO:3 and retain its activity are conventional in the art." Likewise, procedures for making DNA molecules which are at least 90% homologous to SEQ ID NO:1 and which encode proteins that retain the activity of AD7c-NTP are conventional in the art. As stated in the specification, DNA molecules which are at least 90% homologous to SEQ ID NO:1 may be isolated from cDNA libraries of humans and animals by hybridization under stringent conditions to the DNA molecule of SEQ ID NO:1 according to methods known to those of skill in the art. *See* specification at page 19, lines 3-15. Applicants note that many other methods for obtaining DNA molecules that are included within the presently claimed invention were well known to persons having ordinary skill in the art at the time of the invention; examples include random and directed mutagenesis of a DNA molecule to produce a variant of SEQ ID NO:1 that is at least 90% homologous thereto. In addition, proteins encoded by variants of SEQ ID NO:1 can easily be tested for AD7c-NTP activity using the procedures described in the specification (*see*

discussion immediately below) as well as with other methods that are conventional in the art for testing the biological activity of a protein.

Fourth, in Example 14 of the Written Description Guidelines, it is stated that "an assay is described [in the specification] which will identify other proteins having the claimed catalytic activity." Correspondingly, in Applicants' specification, assays are described which will identify other DNA molecules encoding proteins having an activity of AD7c-NTP. For example, the specification describes the production of transgenic animals which over-express AD7c-NTP and the analysis of such animals for "evidence of neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and gliblastomas." *See* specification at page 20, lines 1-29. (The ability of one skilled in the art to produce transgenic animals is described in more detail below). The specification also describes an *in vitro* assay for AD7c-NTP activity involving the over-expression of AD7c-NTP in neuronal cells and the analysis of such cells for growth properties and morphology, including the occurrence of apoptosis and neuritic sprouting. *See* specification at page 45, line 16, through page 46, line 26.

As demonstrated above, the hypothetical situation described in Example 14 of the USPTO's Written Description Synopsis is very similar to the situation presented for the DNA molecules used with, or found within the subject matter encompassed by Applicants' currently-presented claims. Since the USPTO guidelines conclude that adequate written description is provided for the hypothetical claim in Example 14, it follows that there is adequate written description for the DNA molecules used with, or found within, Applicants' currently-presented claims.

Applicants' contention that the written description requirement is satisfied for DNA molecules which are at least 90% homologous to SEQ ID NO:1 is supported, not only by the USPTO's Written Description Synopsis, but also by the Federal Circuit's interpretation and application of 35 USC § 112, first paragraph. *See, e.g., Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997). According to the Federal Circuit, the disclosure of a patent must allow one skilled in the art to visualize or recognize the identity of the subject matter of the claim. *See id.* at 1568, 43 USPQ2d at 1406. Applicants have provided in the specification a detailed analysis of the sequence characteristics of the AD7c-NTP cDNA and of the corresponding translated amino acid sequence. *See* specification at page 34, line 6, through page 35, line 28. Applicants have also described various activities possessed by proteins encoded by AD7c-NTP and methods for assaying such activities. *See, e.g.*, specification at page 46, lines 4-26. Moreover, methods for making DNA molecules that are 90% homologous to a reference DNA molecule are common in the field of molecular biology and are also described in the specification. *See, e.g.*, specification at page 19, lines 3-15. In view of these factors, a skilled artisan would be able to clearly visualize and recognize the DNA molecules that are used with, or found within the subject matter encompassed by Applicants' currently-presented claims.

2. A Transgenic Animal Comprising A DNA Molecule Which is At Least 40% Homologous to SEQ ID NO:1 and Its Corresponding Phenotype

The Examiner's second basis for the written description rejection is the contention that "the specification does not provide sufficient description of any transgenic animal

comprising a sequence with at least 40% homology to SEQ ID NO:1 and its corresponding phenotype." *See* Paper No. 12, page 4. The Examiner further stated:

> Claiming unspecified transgenic animals comprising a nucleotide sequence with at least 40% homology to the nucleotide sequence set forth in SEQ ID NO:1 that must possess the biological properties as contemplated by applicant's disclosure without defining what means will do so is not in compliance with the written description requirement. . . . The skilled artisan cannot envision the detailed structure of a genus of transgenic animals comprising a nucleotide sequence which is at least 40% homolog[ous] to SEQ ID NO:1 or any phenotype that must exhibit the contemplated biological functions, and therefore, conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the structures and/or methods disclosed in the as-filed specification.

Paper No. 12, page 5.

Applicants respectfully traverse this basis of rejection. Nevertheless, solely to expedite prosecution, claim 7 has been amended to recite "a DNA molecule which is at least 90% homologous thereto," rather than "a DNA molecule which is at least 40% homologous thereto." Accordingly, this basis of rejection, insofar as it relates to the inclusion in the claims of DNA molecules that are at least 40% homologous to SEQ ID NO:1, is moot.

With respect to the Examiner's statements relating to the ability of a skilled artisan to envision "any *phenotype* that must exhibit the contemplated biological functions," Applicants note that the claims do not specify any particular phenotype that the transgenic animals of the invention must possess. Moreover, claim 7 has been amended to make explicit the implicit requirement that the DNA molecule of SEQ ID NO:1, or a DNA molecule that is at least 90% homologous thereto, is expressed in one or more cells of the transgenic animal. As discussed elsewhere in this Reply, the transgenic animals of the

invention are useful for, among other things, screening for drugs that can be useful for the treatment or prevention of Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas, and glioblastomas. *See* specification at page 21, lines 3-6. It would be appreciated by one of ordinary skill in the art that transgenic animals of the invention -- whose cells express the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto -- would be useful in the contemplated screening methods of the invention regardless of whether such animals display any obvious phenotype; such transgenic animals can, for example, be used to screen for drugs that suppress or prevent the expression of the protein coded by the DNA molecule, or to screen for drugs that cause increased degradation of the protein. *See* specification at page 21, lines 11-13. Thus, in terms of satisfying the written description requirement of 35 USC § 112, first paragraph, it is not necessary that a skilled artisan be able to envision any particular phenotype that the claimed transgenic animals may or may not possess.

3. Summary

Applicants have conveyed with reasonable clarity to those skilled in the art that, as of the effective filing date, they were in possession of the presently-claimed transgenic animals and methods of screening which involve the use of such transgenic animals. Therefore, under *Vas-Cath,* 935 F.2d at 1560, 19 USPQ2d at 1117, Applicants have fully satisfied the written description requirement of 35 USC § 112, first paragraph. Applicants respectfully request that the rejection of claims 7, 9, 14 and 16 under § 112, first paragraph, for lack of adequate written description, be reconsidered and withdrawn.

B. Enablement

The Examiner has rejected claims 7-9 and 14-16 under 35 USC § 112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. *See* Paper No. 12, page 6. Applicants respectfully traverse the rejection.

There are three distinct bases that the Examiner has set forth in support of the rejection for lack of enablement. The first basis is directly related to the Examiner's rejection for alleged insufficient written description. *See* Paper No. 12, page 6. The second basis relates to the enablement provided for making and/or using DNA molecules that are at least 40% homologous to SEQ ID NO:1. *See* Paper No. 12, pages 6-8. The third basis relates to the enablement provided for making and/or using transgenic animals expressing a nucleotide sequence encoding SEQ ID NO:1 or a DNA molecule that is at least 40% homologous thereto. *See* Paper No. 12, pages 8-13. Each of these bases for the rejection is addressed in turn below.

1. Written Description of the Claimed Invention

The first basis for the enablement rejection is the Examiner's position that the specification does not provide adequate written description of the claimed invention. In particular, the Examiner stated:

> since the claimed invention is not supported by a sufficient written description (for possession of a genus of a transgenic animal comprising a nucleotide sequence comprising a DNA molecule which is at least 40% homologous to SEQ ID NO:1 and its corresponding phenotype), particularly in view of the

> reasons set forth above, one skilled in the art would not have known how to use and make the claimed invention so that it would operate as intended, e.g. for use in a method for testing potential Alzheimer's Disease drugs.

See Paper No. 12, page 6.

As discussed above, the specification provides adequate written description for the subject matter encompassed by Applicants' currently presented claims. A person of ordinary skill in the art, based on the specification, would appreciate that Applicants' were in possession of the claimed subject matter. Therefore, the first basis for the enablement rejection has been fully addressed by Applicants' comments presented above.

2. Enablement Provided for the DNA Molecules That are Included Within the Transgenic Animals of the Invention

The second basis for the enablement rejection is the Examiner's contention that "the as-filed specification does not provide sufficient guidance for one skilled in the art to make and/or use any DNA molecule which is at least 40% homologous to SEQ ID NO:1." *See* Paper No. 12, page 6.

Applicants respectfully disagree with the Examiner's assertion that DNA molecules that are 40% homologous to SEQ ID NO:1 are not enabled by the present specification. Nevertheless, solely to expedite prosecution, Applicants have replaced the expression "40% homologous" with "90% homologous" in claim 7. Thus, the Examiner's comments, insofar as they are directed to DNA molecules that are 40% homologous to SEQ ID NO:1, are moot.

The Examiner, in explaining this basis for rejection, has focused on the asserted "unpredictability of the relationship between [amino acid] sequences and [protein] function." *See* Paper No. 12, page 7. More specifically, the Examiner stated that:

> the as-filed specification does not provide sufficient guidance for how one skilled in the art would be enabled to reasonably correlate SEQ ID NO:1 to a nucleic acid which is at least 40% homologous to SEQ ID NO:1, since at the time the application was filed, predicting any protein tertiary structure based on a protein structure was considered to be unpredictable due to significant problems in several areas.

Paper No. 12, pages 6-7. To support this position, the Examiner cited an excerpt from Chiu *et al.*, "Folding and Design," Vol. 3 (1998), describing the difficulties associated with predicting the three-dimensional conformation of a correctly folded protein. *See* Paper No. 12, page 7.

The relationship between the sequence of a protein and its biological function may in fact be complex, and it may be difficult to predict the exact functional consequences of a particular mutation. However, a skilled artisan would not need to be able to predict the structural and/or functional consequences of particular mutations or base changes in order to produce DNA molecules that are 90% homologous to SEQ ID NO:1 and that code for proteins having an activity of AD7c-NTP. To make the DNA molecules that are included within the transgenic animals of the present invention, the skilled artisan would only need to be able to: (a) obtain DNA molecules that are at least 90% homologous to SEQ ID NO:1, and (b) test them for the ability to encode proteins that possess AD7c-NTP activity. As discussed immediately below, both of these processes would be routine in the art.

A person of ordinary skill in the art would be able to generate a DNA molecule which is at least 90% homologous to SEQ ID NO:1 using methods that are routine in the art. The specification provides exemplary methods for obtaining DNA molecules which are at least 90% homologous to SEQ ID NO:1; such methods involve the isolation of DNA molecules from cDNA libraries by hybridization under stringent conditions to the DNA

molecule of SEQ ID NO:1. *See* specification at page 19, lines 3-15. Additional methods for obtaining DNA molecules for use with the claimed invention (*i.e.*, DNA molecules that are at least 90% homologous to SEQ ID NO:1), include the use of directed and random mutagenesis techniques; such methods were well known to those of ordinary skill in the art at the time of the invention.

Once obtained, DNA molecules that are at least 90% homologous to SEQ ID NO:1 can easily be tested for the ability to encode a protein having an activity of AD7c-NTP. The specification describes various methods for assaying for AD7c-NTP activity. For example, transgenic animals can be made that over-express AD7c-NTP, and, once obtained, the transgenic animals may be analyzed for evidence of neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. *See* specification at page 20, lines 1-29. (The ability of a person of ordinary skill in the art to produce transgenic animals whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto is discussed in more detail below). Additionally, *in vitro* methods can be used to test for AD7c-NTP activity. For example, the specification describes an assay involving the overexpression of AD7c-NTP in neuronal cells and the subsequent analysis for cellular characteristics of Alzheimer's disease, including apoptosis and neuritic sprouting. *See* specification at page 46, lines 4-26. Thus, the full range of DNA molecules that are included within the transgenic animals of the invention can be easily made and analyzed by persons of ordinary skill in the art using only routine methods and experimentation.

Of course, it is possible that DNA molecule that are at least 90% homologous to SEQ ID NO:1 but that *do not* encode proteins with AD7c-NTP activity may be identified by the

methods described above. The skilled artisan, however, would be able to easily identify and discard such non-active molecules. Screening for molecules that possess a particular activity is common in the biological arts. Experimentation, even complex experimentation, is not undue if the art typically engages in such experimentation. *See In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985); *see also Wands*, 858 F.2d at 737, 8 USPQ2d at 1404.

Thus, any uncertainty that is associated with predicting protein function from sequence data is of little relevance in an analysis of the enablement of Applicants' claims. A skilled artisan would be expected to engage in screening for DNA molecules that are at least 90% homologous to SEQ ID NO:1 and that encode proteins having AD7c-NTP activity when expressed in neuronal cells. Such screening, even if it resulted in the identification of molecule not having the desired activity, would be considered routine in the art.

The Examiner has cited Altieri *et al.*, U.S. Patent No. 6,245,523, and Frangiskakis *et al.*, *Cell 86*:59-69 (1996), as allegedly disclosing nucleotide sequences that are 74.9% and 40.8% homologous to SEQ ID NO:1, respectively. *See* Paper No. 12, pages 7-8. The Examiner stated that the nucleotide sequence described in Altieri "inhibits cellular apoptosis," and that the nucleotide sequence described in Frangiskakis encodes a protein kinase. *See* Paper No. 12, pages 7-8.

With respect to the nucleotide molecules disclosed in Altieri and Frangiskakis, Applicants first note that the present claims do not include or encompass transgenic animals comprising nucleotide molecules that are 74.9% or 40.8% homologous to SEQ ID NO:1. Moreover, if the nucleotide molecules of Altieri and Frangiskakis encode proteins that in

fact do *not* have AD7c-NTP activity (as the Examiner has asserted), then a skilled artisan would easily be able to ascertain that fact using the methods for assaying AD7c-NTP activity that are taught in the specification and that would be known to persons having ordinary skill in the art. Thus, the Examiner's discussion of the nucleotide molecules of Altieri and Frangiskakis does not provide evidence tending to cast doubt on the enablement of Applicants' claimed invention.

In view of the foregoing discussion, Applicants submit that the specification fully enables the making and using of DNA molecules that are at least 90% homologous to SEQ ID NO:1 and that encode proteins that have an activity of AD7c-NTP when expressed in neuronal cells.

3. Enablement Provided for the Transgenic Animals That are Encompassed by or Used in the Practice of the Claimed Invention

The third basis for the enablement rejection relates to the level of guidance provided in the specification for the production of transgenic animals. The Examiner's position is that "the as-filed specification does not provide sufficient guidance or factual evidence for any transgenic animal expressing a nucleotide sequence encoding SEQ ID NO: 1 or a DNA molecule, which is at least 40% homologous thereto, and any corresponding phenotype." *See* Paper No. 12, page 9. Applicants respectfully disagree with this contention.

Applicants first note that claim 7 has been amended to replace the expression "40% homologous" with "90% homologous." Thus, the third basis of rejection, insofar as it relates to the inclusion of DNA molecules that are 40% homologous to SEQ ID NO:1, is moot.

The Examiner stated that "the as-filed specification only contemplates the use of embryonic stem (ES) cell technology or using pro-nuclear injection for the generation of transgenic mammals for [use] in the claimed invention." *See* Paper No. 12, page 9. The Examiner cited page 20 of the specification to support this assertion. Applicants respectfully disagree. Applicants note that ES cell technology and pronuclear microinjection are only two exemplary methods that can be used to produce the transgenic animals of the present invention. For instance, the specification states that "transgenic animals *may* be obtained, *for example*, by injecting the DNA construct of the invention into a fertilized egg which is allowed to develop into an adult animal." *See* specification at page 20, lines 3-5 (emphasis added).

It is also clear from the specification that any method known to those skilled in the art can be used for the production of the transgenic animals of the invention. For example, the specification at page 20, lines 18-19, cites, among other references, U.S. Patent No. 5,602,299 ("Lazzarini") (incorporated by reference in the specification in its entirety and included herewith as Exhibit A). This reference is cited in the specification as providing examples of methods of preparing transgenic animals. Lazzarini states:

> Any technique known in the art may be used to introduce the transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection; retrovirus mediated gene transfer into germ lines; gene targeting in embryonic stem cells; electroporation of embryos; and sperm-mediated gene transfer; etc.

See U.S. Patent No. 5,602,299 at column 13, lines 2-12 (internal citations omitted). Thus it is incorrect to state that the specification *only* contemplates the use of ES cell technology and pronuclear injection to produce transgenic animals.

Moreover, an Applicant is not limited to the confines of the specification to provide the necessary information to enable an invention. *See In re Howarth*, 654 F.2d 103, 105-6, 210 USPQ 689, 692 (CCPA 1981). An Applicant need not supply information that is well known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997); *Howarth*, 654 F.2d at 105-6, 210 USPQ at 692; *see also In re Brebner*, 455 F.2d 1402, 173 USPQ 169 (CCPA 1972) (finding a disclosure enabling because the procedure for making the starting material, although not disclosed, would have been known to one of ordinary skill in the art as evidenced by a Canadian patent). "'That which is common and well known is as if it were written out in the patent and delineated in the drawings.'" *Howarth*, 654 F.2d at 106, 210 USPQ at 692 (quoting *Webster Loom Co. v. Higgins* et al., 105 U.S. (15 Otto.) 580, 586 (1881)). Additionally, one of ordinary skill in the art is deemed to know not only what is considered well known in the art but also where to search for any needed starting materials. *Id.* Thus, in order to generate the transgenic animals of the present invention, a person of ordinary skill in the art would have at his or her disposal any method of transgenic animal production that was known in the art at the time of the application.

Despite the fact that multiple methods for producing transgenic animals were available to those skilled in the art at the time the application was filed, the Examiner, in explaining the enablement rejection, has focused his attention on only two exemplary methods: pronuclear microinjection and embryonic stem cell technology. This analysis is incomplete since many other methods were available to the skilled artisan. The Examiner has not set forth any evidence or sound scientific reasoning that would indicate that other methods for producing transgenic animals would require undue experimentation. Moreover,

the Examiner has not provided evidence that would indicate that transgenic animal production using pronuclear microinjection or embryonic stem cell technology would entail a degree of experimentation that would be regarded as undue in the context of the present invention.

With respect to the technique of pronuclear microinjection, the Examiner asserted that "[t]he state of the art at the time [the] application was filed for producing transgenic animals using pro-nuclear injection was considered unpredictable as exemplified by Polejaeva et al. Theriogenology, Vol. 53, pages 117-126, 2000." *See* Paper No. 12, page 9. To support this proposition, the Examiner specifically cited Polejaeva at page 119. Here, Polejaeva begins by acknowledging that "[t]ransgenic animals *can be successfully produced in a number of species* including mice, rabbits, pigs, sheep, cattle and goats by the injection of the gene of interest into the pronucleus of a zygote." *See* Polejaeva at page 119, second full paragraph (emphasis added, internal citations omitted). Thus, the first sentence of the cited passage of Polejaeva directly refutes the assertion that transgenic animal production using pronuclear microinjection would entail undue experimentation.

Polejaeva goes on to describe the "limitations" that are associated with pronuclear injection. The "most profound limitation," mentioned by Polejaeva, is that DNA can only be added, not deleted or modified *in situ*. *See id.* Since the production of transgenic animals that are included within Applicants' claims only requires the addition of DNA molecules, this "most profound limitation" is inapplicable to Applicants' invention.

The other two limitations of pronuclear microinjection cited by Polejaeva are (a) the potential for random integration of foreign DNA, and (b) the possibility of creating mosaic

animals. *See id.* In view of the potential for random integration into an animal's genome using pronuclear microinjection, the Examiner asserted that:

> . . . it would take one skilled in the art an undue amount of experimentation to reasonably extrapolate from random integration to determining if a DNA sequence set forth in SEQ ID NO:1 is inserted at the correct site and is expressed at a level sufficient enough to produce a phenotype in any transgenic non-human animal.

Paper No. 12, pages 10-11.

Aside from citing Polejaeva for the proposition that random integration and the production of mosaics may be a "limitation" in the practice of pronuclear microinjection to produce transgenic animals, the Examiner has not set forth any evidence that would suggest that this method would require an "undue amount of experimentation." The potential limitations associated with pronuclear microinjection only indicate that a certain level of experimentation may be needed to create transgenic animals using this technology. The necessity of some experimentation to practice a claimed invention does not render an invention non-enabled as long as the quantity of experimentation needed is not regarded as undue. *See In re Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219 (CCPA 1976).

There is no indication that the experimentation needed to successfully obtain transgenic animals using pronuclear microinjection would be regarded as *undue*. Indeed, the fact that transgenic animals can be, and have been, successfully produced using pronuclear microinjection indicates that the practice of this technology is not regarded as requiring undue experimentation. *See* Polejaeva at page 119, second full paragraph. In addition, others in the field have noted that:

> Pronuclear DNA injection has enabled the scientific community world wide to selectively add defined genes of

> choice into the germ line of laboratory as well as farm animals. Many experiments with transgenic animals confirmed that transgenesis can provide new insight into many aspects of mammalian life, development and diseases.

See Rülicke and Hübscher, *Exp. Physiol. 85*:589-601 (2000) at page 597, left column, first full paragraph.

In addition, it appears that the Examiner has inadvertently misquoted an important sentence from Polejaeva. The Examiner quotes Polejaeva as stating: "Therefore, the production of the required phenotype coupled to germ line transmission could [require (?)] undue experimentation." *See* Paper No. 12, page 9. The actual quote is as follows: "Therefore, the production of the required phenotype coupled to germ line transmission could require the generation of several transgenic founder lines." *See* Polejaeva at page 119, second full paragraph. Far from indicating that the use of pronuclear injection involves "undue experimentation," Polejaeva merely notes that the "limitations" associated with this methodology may require that several transgenic founder lines be produced. There is nothing to suggest that producing multiple founder lines is of such difficulty as to satisfy the legal standard of "undue experimentation."

Moreover, the final two sentences in the cited paragraph of Polejaeva indicate that alternative methods are available for producing transgenic animals that may avoid the limitations of pronuclear injection:

> Somatic cell nuclear transfer [described on page 120 of Polejaeva] will eliminate this problem and accelerate transgenic herd or flock generation. In addition, transgenic sheep produced using this new technology require the use of fewer than half the animals needed for pronuclear microinjection.

See Polejaeva at page 119, second full paragraph. Therefore, Polejaeva indicates that somatic cell nuclear transfer (a method that would have been available to one of ordinary skill in the art at the time of the application) is another method that is likely to be successful in the production of transgenic animals.

The Examiner next turned his attention to ES cell technology. The Examiner asserted that ES cell technology "is generally limited to the mouse system." *See* Paper No. 12, page 9. To support this position, the Examiner cited Rülicke and Hübscher, *Exp. Physiol. 85*:589-601 (2000) and Bishop, J.O., *Reprod. Nutr. Dev. 36*:607-618 (1996). *See* Paper No. 12, page 10. Even if it is true that ES cell technology has only been shown to be effective in the production of transgenic mice, Applicants emphasize that many other methods for producing non-mouse transgenic animals were available to those skilled in the art as of the effective filing date of the application. *See, e.g.*, U.S. Patent No. 5,602,299 (Lazzarini), column 12, line 65, through column 13, line 15.

The Examiner stated that "the as-filed specification fails to describe any particular phenotype exhibited by any contemplated transgenic animal of the invention." *See* Paper No. 12, page 11. The Examiner further stated that "the claimed transgenic mammal [sic: animal] is not limited to the expression of the protein at a level resulting in a specific phenotype," but that the broadest interpretation of the claims is such that they encompass a:

> transgenic mammal [sic: animal] having cells, which harbor a recombinant nucleic acid that expresses the protein at a level sufficient to result in a specific phenotype (i.e., it is unknown what other purpose the transgenic mammal [sic: animal] would serve if the transgene (e.g. SEQ ID NO:1 or a sequence with 40% homology thereto) is not expressed at a sufficient level for a resulting phenotype).

See Paper No. 12, page 11. Applicants respectfully disagree with this analysis.

Transgenic animals of the present invention, even if they do not exhibit a particular phenotype, would nonetheless be useful in drug screening applications. One manner by which drugs useful in the treatment or prevention of Alzheimer's disease can be identified is by administering candidate drugs to a transgenic animal whose germ and somatic cells comprise the DNA molecule of SEQ ID NO:1 or a DNA molecule which is at least 90% homologous thereto, and identifying those drugs that cause, *e.g.*, the suppression or prevention of expression of the protein encoded by the DNA molecule contained by the transgenic animal. *See* specification at page 21, line 12. Alternatively, drugs can be identified on the basis of their ability to increase the degradation of the protein encoded by the DNA molecule contained by the transgenic animal. *See* specification at page 21, line 13. Therefore, the only characteristic that the transgenic animals of the invention need to possess in order to be useful for the contemplated screening methods is that they express the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto. Accordingly, claim 7 has been amended to specify that the DNA molecule is expressed in one or more cell of said transgenic animal. Thus, contrary to the Examiner's assertion, it would be clear what purpose transgenic animals of the invention would serve even if they did not exhibit a phenotype caused by the DNA molecule: they would be useful for screening for drugs that influence transgene expression.

Even though it is not necessary that the transgenic animals of the invention exhibit any particular phenotype, various phenotypes are nonetheless described in the specification. For example, the specification at page 20, lines 26-29 indicates that:

> Once obtained, the transgenic animals which contain the AD7c-NTP may be analyzed by immunohistology for evidence of AD7c-NTP expression as well as for evidence of

> neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas.

In addition, in discussing the use of the transgenic animals of the invention for screening applications, the specification states that drug candidates can be tested by contacting them with a host (*e.g.*, a transgenic animal) transfected with a DNA construct comprising the DNA molecule of SEQ ID NO:1 or a DNA molecule that is at least 90% homologous thereto. *See* specification at page 21, lines 7-10. Drug candidates can be identified, *e.g.*, on the basis of their ability to reduce the frequency of at least one of neuritic sprouting, nerve cell death, degenerating neurons, neurofibrillary tangles, or irregular swollen neurites and axons in the host. *See* specification at page 21, lines 14-18. Thus, according to the specification, the transgenic animals of the invention may (but need not necessarily) exhibit neuronal or neuritic abnormalities associated with Alzheimer's disease, neuroectodermal tumors, malignant astrocytomas and glioblastomas. More specifically, the phenotypes potentially exhibited by the transgenic animals of the invention, as set forth in the specification, include neuritic sprouting, nerve cell death, nerve cell degeneration, neurofibrillary tangles, and/or irregular swollen neurites.

Applicants' claims do not require that the transgenic animals of the invention exhibit any particular phenotype. The Examiner, however, based much of his arguments in support of the enablement rejection on the assertion that producing transgenic animals with specific phenotypes is unpredictable. *See* Paper No. 12, page 12. For instance, the Examiner stated:

> [O]ne skilled in the art would not be able to rely on the state of the art for an attempt to produce any transgenic animals. This is because of the art of transgenic[s] is not predictable art with respect to transgene behavior and the resulting phenotype. While the state of the art of transgenics is such

> that one of skill in the art would be able to produce transgenic animal comprising a transgene of interest (e.g. SEQ ID NO:1 or a sequence with 40% homology thereto); it is not predictable if the transgene would be expressed at a level and specificity sufficient to cause a particular phenotype.

Paper No. 12, page 12. The Examiner then listed several factors that may be important in controlling the expression of a transgene in transgenic animals. *See id.* To support the assertion that the specific characteristics of a transgene construct are important in the context of producing transgenic animals, the Examiner cited Wall, R.J., *Theriogenology 45*:57-68 (1996) and Houdebine, L-M., *J. Biotechnology 34*:269-287 (1994). *See* Paper No. 12, pages 12-13. The Examiner also noted that the expression of a transgene in one species is not always indicative of the level of expression of the same transgene in another species. *See* Paper No. 12, page 13. To support this assertion, the Examiner cited Mullins, L.J. and Mullins, J.J., *J. Clin. Invest. 97*:1557-1560 (1996) and Strojek, R.M. and Wagner, T.E., *Genetic Engineering: Principles and Methods 10*:221-246 (1988). In view of these references, the Examiner concluded:

> it would require an undue amount of experimentation to reasonably predict the results achieved in any transgenic mammal comprising a transgenic sequence set forth in SEQ ID NO:1 or a sequence with 40% homology thereto and which over-expresses the protein in the transgenic animal at the levels of the claimed product, the consequences of that production, and therefore, the resulting phenotype.

Paper No. 12, page 13. In view of the fact that the present claims do not require that the transgenic animals of the invention exhibit any particular phenotype, the Examiner's remarks, set forth above, do not support a rejection under 35 USC § 112, first paragraph.

Furthermore, Applicants respectfully disagree with the Examiner's conclusion regarding the ability of one skilled in the art to produce transgenic animals with a desired phenotype.

The references cited by the Examiner, rather than demonstrating that the production of transgenic animals with a particular phenotype requires undue experimentation, merely indicate that certain technical issues should be considered in order to successfully produce transgenic animals exhibiting a certain phenotype. To establish a *prima facie* case of non-enablement, it is not sufficient for an Examiner to show that *some* experimentation may be required to make and use the claimed invention; the Examiner must present evidence indicating that *undue* experimentation is required. *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971), *see also In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). The references cited by the Examiner simply describe certain challenges in the art. None of these references suggest that such challenges are insurmountable or that the production of transgenic animals with certain phenotypes would require undue experimentation.

In fact, the references cited by the Examiner actually support the position that producing transgenic animals with a particular phenotype *does not* involve a degree of experimentation that would be regarded as undue in the art. The cited references describe several instances in which transgenic animals exhibiting a desired phenotype were successfully produced. Wall, for example, summarizes the results of various researchers demonstrating the production of: (i) transgenic sheep with enhanced wool production characteristics, *see* Wall at page 59, third full paragraph; (ii) transgenic mice that serve as models for human genetic diseases (including Alzheimer's disease), *see id.*; and (iii) transgenic pigs that express a human complement inhibitor for use in xenograft

mice and a transgenic pig that expressed the human complement inhibitor hCD59, the cells of which exhibited resistance to challenge with high-titer anti-porcine antibody and human complement, *see* Fodor, W.L. *et al.*, *Proc. Natl. Acad. Sci. USA 91*:11153-11157 (1994) (included herewith as Exhibit D), *see* especially page 11157, left column, last full paragraph; and (iv) the production of a transgenic pig that expressed a murine leukemia virus-rat somatotropin fusion gene and that produced high levels of circulating rat somatotropin and exhibited increased skeletal growth and reduced fat deposition, *see* Ebert, K.M. *et al.*, *Mol. Endocrinol. 2*:277-283 (1988) (included herewith as Exhibit E), *see* especially paragraph bridging pages 280-281.

The fact that there are numerous examples in the art of successfully produced transgenic animals expressing specific desired phenotypes, indicates that the production of such transgenic animals does not require undue experimentation.

4. Summary

Applicants assert that the transgenic animals encompassed by, and used in the practice of the presently claimed invention, could be produced by one of ordinary skill in the art with only routine experimentation based on the teachings of the specification and the knowledge generally available in the art. Therefore, Applicants respectfully request that the rejection of claims 7-9 and 14-16 under 35 USC § 112, first paragraph, for lack of enablement, be reconsidered and withdrawn.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

Frank R. Cottingham

Frank R. Cottingham
Attorney for Applicants
Registration No. 50,437

Date: 8/08/02

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

Version with markings to show changes made

In the Specification:

At page 1, immediately after line 3 and before line 4 (*i.e.*, between the title of the invention and the heading labeled "Background of the Invention") please insert the following caption and paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a divisional of U.S. patent application No. 09/380,203, filed April 25, 2000, which is a 371 of PCT/US98/03685, filed February 26, 1998 and published under PCT Article 21(2) in English on September 3, 1998, which claims the benefit of the filing date of U.S. provisional patent application No. 60/038,908, filed February 26, 1997.

In the Claims:

Please substitute the following claim 7 for the pending claim 7:

7. (Once amended) A transgenic non-human animal, all of whose germ and somatic cells comprise[s] the DNA molecule of [Seq. ID No. 1] SEQ ID NO:1 or a DNA molecule which is at least [40%] 90% homologous thereto, wherein said DNA molecule is expressed in one or more cells of said transgenic animal, and wherein said DNA molecule codes for a protein that has an activity of AD7c-NTP when expressed in neuronal cells.

Please substitute the following claim 8 for the pending claim 8:

8. (Once amended) The transgenic non-human animal of claim 7, wherein the DNA molecule contained in each germ and somatic cell has [Seq. ID No. 1] SEQ ID NO:1.

Please substitute the following claim 15 for the pending claim 15:

15. (Once amended) The method of claim 14, wherein the DNA construct contained by said animal has [Seq. ID No. 1] SEQ ID NO:1.

Please add claims 35 and 36.

EXHIBIT A

US005602299A

United States Patent [19]

Lazzarini

[11] Patent Number: 5,602,299

[45] Date of Patent: Feb. 11, 1997

[54] **TRANSGENIC ANIMAL MODELS FOR NEURODEGENERATIVE DISEASE**

[75] Inventor: **Robert A. Lazzarini**, New York, N.Y.

[73] Assignee: **Mount Sinai School of Medicine of the City University of New York**, New York, N.Y.

[21] Appl. No.: **950,092**

[22] Filed: **Sep. 23, 1992**

[51] **Int. Cl.**[6] **A01K 67/00**; C12N 15/00

[52] **U.S. Cl.** **800/2**; 800/DIG. 1; 435/172.3; 435/320.1; 536/23.5

[58] **Field of Search** 800/2; 435/172.1, 435/172.3, 173; 935/6, 9; 536/23.1, 23.5, 24.1; 424/9

[56] **References Cited**

PUBLICATIONS

Geisler, N. and Weber, K., 1981, J. Mol. Biol. 151: 565–571.
Sharp, G. A. et al., 1982, Exp. Cell Res. 137:403–413.
Liem, R. K. H. and Hutchinson, S. B., 1982, Biochemistry 21:3221–3226.
Julien, J.–P. and Mushynski, W. E., 1983, J. Biol. Chem. 258:4019–4025.
Portier, M. M. et al., 1984, Dev. Neurosci. 6:335–344.
Pachter, J. S. and Leim, R. K. H., 1984, Dev. Biol. 103:200–210.
Hirokawa, N. M. et al., 1984, J. Cell Biol. 98:1523–1536.
Hoffman, P. N., et al., 1984, J. Cell Biol. 99:705–714.
Bennett, G. S. and DiLullo, C., 1985, Dev. Biol. 107:107–127.
Pierre, M. et al., 1985, Ann. N.Y. Acad. Sci. 495:808–811.
Carden, M. J. et al., 1985, J. Biol. Chem. 260:9805–9817.
Lee, V. M.–Y. et al., 1986, J. Neurosci. 6:2179–2186.
Levy, E. et al., 1987, Eur. J. Biochem. 166:71–77.
Kang, J. et al., 1987, Nature 325:733–736.
Kollias, G. et al., 1987, Proc. Natl. Acad. Sci. USA 84:1492–1496.
Julien, J.–P. et al., 1987, Biochem. Biophys. Acta 909:10–20.
Julien, J.–P., et al., 1987, Genes and Dev. 1:1085–1095.
Myers, M. W. et al., 1987, EMBO J. 6:1617–1626.
Gordon, J. et al., 1987, Cell 50:445–452.
Lee, V. M.–Y. et al., 1987, J. Neurosci. 7:3474–3488.
Leonard, D. G. B. et al., 1988, J. Cell Biol. 106:181–193.
Lee, V.–M. Y. et al., 1988, Proc. Natl. Acad. Sci. USA 85:1998–2002.
Lees, J. F. et al., 1988, EMBO J. 7:1947–1955.
Steinert, P. M. and Roop, D. R., 1988, Ann. Rev. Biochem. 57:593–625.
Hisanaga, S.–I. and Hirokawa, N., 1988, J. Mol. Biol. 202:297–305.
Julien J.–P. et al., 1988, Gene 68:307–314.
Wible, B. A. et al., 1989, Proc. Natl. Acad. Sci. USA 86:720–724.
Pleasure, S. J. et al., 1989, J. Neurosci. 9:698–709.
Szaro, B. G. et al., 1989, Dev. Brain Res. 48:87–103.
Hyman, B. T. et al., 1989, Neurosci. Lett. 101:352–355.
Evans, D. A. et al., 1989, JAMA 262:2551–2556.
Lendahl, U. L. et al. 1990, Cell 60:585–595.
Fliegner, K. H. et al., 1990, EMBO J. 9:749–755.
Vidal, M. et al., 1990, EMBO J. 9:833–840.
Pleasure, S. J. et al., 1990, J. Neurosci. 10:2428–2437.
Forss–Petter, S. et al., 1990, Neuron 5:187–197.
Montiero, M. J., 1990, J. Cell Biol. 111:1543–1557.
Shaw, C., 1991, Neurofilament Proteins, In "The Neuronal Cytoskeleton", Burgoyne, R. R., ed., pp. 185–214, Wiley–Liss, Inc., N.Y.
Balin, B. J. et al., 1991, Brain Res. 556:181–195.

(List continued on next page.)

Primary Examiner—Jacqueline M. Stone
Assistant Examiner—Bruce Campbell
Attorney, Agent, or Firm—Pennie & Edmonds

[57] **ABSTRACT**

The design, construction, and use of transgenic animals which exhibit features, including neurofibrillary tangles and aluminum sensitivity, is described. The founder transgenic animals of the invention are produced by methods well known in the art, and utilize DNA sequences designed to express all or any part of the human neurofilament subunit genes, NF-L, NF-M, NF-H, in a neural-enriched manner.

3 Claims, 19 Drawing Sheets

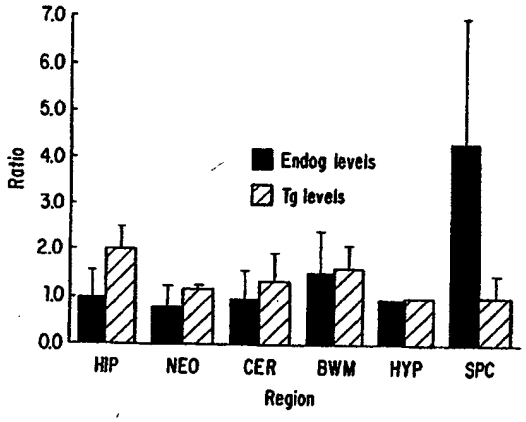


EXHIBIT B

Cell, Vol. 63, 1099–11 ovember 30, 1990, Copyright © 1990 by Cell Press

Spontaneous Inflammatory Disease in Transgenic Rats Expressing HLA-B27 and Human β_2m: An Animal Model of HLA-B27-Associated Human Disorders

Robert E. Hammer,*† Shanna D. Maika,* James A. Richardson,‡ Jy-Ping Tang,§ and Joel D. Taurog§
* Howard Hughes Medical Institute
†Department of Biochemistry
‡Department of Pathology
Division of Comparative Medicine
§Harold C. Simmons Arthritis Research Center
and Department of Internal Medicine
University of Texas Southwestern Medical Center
Dallas, Texas 75235

Summary

Humans who have inherited the human class I major histocompatibility allele HLA-B27 have a markedly increased risk of developing the multi-organ system diseases termed spondyloarthropathies. To investigate the role of B27 in these disorders, we introduced the B27 and human β_2-microglobulin genes into rats, a species known to be quite susceptible to experimentally induced inflammatory disease. Rats from one transgenic line spontaneously developed inflammatory disease involving the gastrointestinal tract, peripheral and vertebral joints, male genital tract, skin, nails, and heart. This pattern of organ system involvement showed a striking resemblance to the B27-associated human disorders. These results establish that B27 plays a central role in the pathogenesis of the multi-organ system processes of the spondyloarthropathies. Elucidation of the role of B27 should be facilitated by this transgenic model.

Introduction

Class I major histocompatibility (MHC) gene products are polymorphic 44,000 M_r glycoproteins expressed on cell surfaces in noncovalent association with the nonpolymorphic 12,000 M_r light chain β_2-microglobulin (Klein, 1986). Among class I MHC molecules, HLA-B27, a serologically defined allele of the human *HLA-B* locus, is of particular interest because it is uniquely associated with a group of relatively common inflammatory disorders. The strongest association is seen with primary ankylosing spondylitis, a chronic inflammatory disease affecting the axial musculoskeletal system: ~90% of affected individuals have inherited the B27 allele in comparison with only ~7% of Caucasians in the general population (Brewerton et al., 1973; Schlosstein et al., 1973; Tiwari and Terasaki, 1985). An important association also exists between HLA-B27 and reactive arthritis, in which certain microbial infections of the gastrointestinal or genitourinary tracts trigger inflammation in joints and other tissues (Toivanen and Toivanen, 1988). A summary of the major disorders associated with HLA-B27 is presented in Table 1.

The B27-associated diseases are classified as rheumatic disorders because of the prominence of musculoskeletal manifestations. Nonetheless, all of these diseases can involve multiple organ systems, particularly the gastrointestinal tract, genitourinary tract, skin, eye, and heart. Because of the overlap among these diseases with regard to epidemiology, clinical manifestations, and anatomic pathology, they were recognized as a distinct cluster of interrelated diseases, termed spondyloarthropathies, even before the common genetic marker of B27 was identified (Moll et al., 1974). Thus it has long been speculated that a common pathogenetic mechanism might underlie the association of B27 with this heterogeneous group of disorders. Despite extensive investigation, however, the etiology and pathogenesis of these diseases have remained obscure, and the basis for the association with B27 has not been established.

In an attempt to develop an animal model of B27-associated disease, we (Taurog et al., 1988a) and others (Krimpenfort et al., 1987; Nickerson et al., 1990; Weiss et al., 1990) have produced transgenic mice expressing HLA-B27 and human β_2-microglobulin ($h\beta_2m$). However, despite physiologically normal function of B27 in both hybrid and inbred mice (Kievits et al., 1987; Taurog et al., 1988a) and a reported influence of B27 on the course of an experimental bacterial infection in mice (Nickerson et al., 1990), no faithful reproduction of any of the features of B27-associated human disease has been reported in transgenic mice. These negative results raised the possibility that susceptibility to the spondyloarthropathies might not be related to the B27 gene. Alternatively, other features of the mouse may not have permitted expression of the relevant pathologic changes. We therefore sought to develop transgenic technology in rats, which are susceptible to several experimentally induced arthritic diseases that cannot be elicited in mice (Greenwald and Diamond, 1988).

In this paper, we describe the production of transgenic rats that express HLA-B27 and $h\beta_2m$ genes. We further describe a disorder spontaneously arising in these B27 transgenic rats that includes most of the features of B27-associated disease in humans.

Results

Integration of HLA-B27 and $h\beta_2m$ Genes in Inbred Rats

Fertilized one-cell rat eggs were microinjected with a solution containing both DNA fragments shown in Figure 1. The HLA-B27 gene encoding the HLA-B*2705 subtype was contained on a 6.5 kb EcoRI fragment that included 0.7 kb of 5′ flanking sequence and 2.5 kb of 3′ flanking sequence (Figure 1A). The $h\beta_2m$ gene was contained on a 15 kb SalI–PvuI fragment that included 5.2 kb of 5′ flanking sequence and 1.9 kb of 3′ flanking sequence (Figure 1B). Identification and quantitation of transgenes in the founder animals and their progeny were determined by dot-blot hybridization of genomic DNA isolated from tail bi-

Table 1. Rheumatic Diseases Associated with HLA-B27

Characteristic	Disorder				
	Ankylosing Spondylitis	Reactive Arthritis[a]	Juvenile Spondyloarthropathy	Psoriatic Arthropathy	Enteropathic Arthropathy
Sacroiliitis or spondylitis[b]	100%	<50%	<50%	20%	10%
Peripheral arthritis[c]	25%	90%	90%	95%	90%
Gastrointestinal inflammation	Common, usually asymptomatic	Common, often symptomatic	Not known	Uncommon	All
Skin and nail involvement	Rare	Most	Uncommon	All	Uncommon
Genitourinary involvement (males only)	Uncommon	Most	Uncommon	Uncommon	Rare
Eye involvement[d]	25%	Common	Common	Occasional	Occasional
Cardiac involvement	<5%	5%–10%	Not known, probably rare	Rare	Rare
Usual age of onset (years)	18–40	18–45	7–18	20–50	15–50
Sex prevalence	Males 3:1	Males 3:1[e]	Males 10:1	Equal	Equal
Type of onset	Gradual	Acute	Variable	Variable	Gradual
Role of infectious agents	Unknown	Definite Trigger	Unknown	Unknown	Unknown
Prevalence of HLA-B27[f]	>90%	60%–80%	80%	50%[g]	50%–75%[g]

Table adapted from Calin (1984); Tiwari and Terasaki (1985); Khan and van der Linden (1990); Taurog and Lipsky (1990).
[a] Includes Reiter's syndrome, classically defined as the triad of arthritis, conjunctivitis, and urethritis.
[b] Inflammation in the spine or sacroiliac joints.
[c] Inflammation in joints of the extremities.
[d] Predominantly conjunctivitis in reactive arthritis; iritis with the other disorders.
[e] Male to female ratio is 10:1 if venereally acquired; 1:1 if enteropathically acquired.
[f] Caucasians of northern European extraction only. General prevalence in this population is 6%–8%. Some variation seen in other populations, but the basic associations with HLA-B27 are seen worldwide.
[g] Frequency elevated only in those with spondylitis or sacroiliitis.

opsies. Hybridization was carried out with 5′ and 3′ flanking probes for the HLA-B27 gene (probes A and C in Figure 1A), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

Seven LEW and four F344 rats that developed from microinjected ova showed integration of the HLA-B27 and hβ_2m genes. Of these, four LEW rats and one F344 rat showed cell surface expression of both HLA-B27 and hβ_2m, as assessed by indirect immunofluorescence of peripheral blood lymphocytes (PBLs). One additional LEW rat showed integration and expression of the B27 gene alone. Table 2 summarizes the results of the microinjection experiments.

All of the founder rats expressing the transgenes were subsequently shown to transmit the transgenes to their offspring. One of the six founders, 21-3, was found to be a mosaic, based on non-Mendelian rates of transmission and on enhanced cell surface expression in the offspring. Another founder, 21-4, a female, was shown to have two independently segregating loci of transgene integration, each locus carrying both transgenes. One line arising from this founder, inheriting a locus containing 150 copies of the B27 gene and 90 copies of the hβ_2m gene, was termed 21-4H. The other line, inheriting a locus containing six copies of the B27 gene and six copies of the hβ_2m gene, was termed 21-4L (Table 3).

Lymphocyte Cell Surface Expression of the HLA-B27 and hβ_2m Transgene Products

Expression of the transgene products was estimated by indirect immunofluorescence and flow cytometry of PBLs stained with specific monoclonal antibodies. The relative expression of B27 and hβ_2m in seven transgenic lines is shown in Table 3. To compensate for interexperiment variation, the mean channel fluorescence for each line with each antibody is expressed relative to that determined in the same experiment for PBLs of the transgenic mouse line 56-3, which expresses high levels of both B27 and hβ_2m on PBL surfaces. The highest expression of both gene products was found in the LEW lines 21-4H and 21-4L and the F344 line 33-3.

The patterns of cell surface expression of B27 and hβ_2m in the 21-4H and 21-4L lines are shown in Figures 2A and 2B. The binding of the endogenous rat class MHC I molecules (RT1) to the anti-RT1 antibody OX18 is shown in Figure 2C for both transgenic lines and the nontransgenic control. The levels of expression of B27 and hβ_2m were comparable in the two transgenic lines (Figures 2A

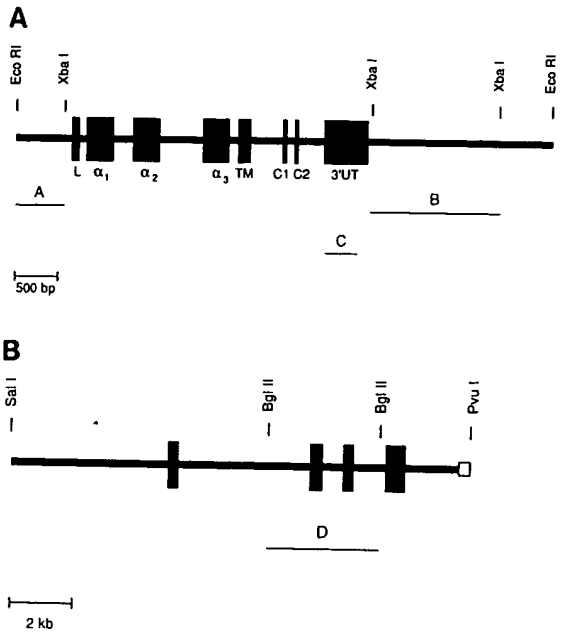


Figure 1. Genes Used for Microinjection of Fertilized Rat Eggs

(A) The HLA-B* 2705 gene (clone pE.1-B27) was contained on a 6.5 kb EcoRI fragment. Exons are indicated by boxes and labeled. Probes from the 5′ and 3′ flanking regions, labeled A and B, respectively, were used for dot-blot hybridization of genomic DNA. Probe C, from the 3′ untranslated region, was used for Northern hybridization.

(B) The hβ2m gene (clone pβ2m-13) was contained on a 15 kb SalI–PvuI fragment. Exons are indicated by boxes. The insert contained ~100 bp of the vector pEMBL9, indicated by the open box at the 3′ end. The 3.7 kb BglII fragment labeled D was used for both dot-blot hybridization of genomic DNA and for Northern hybridization.

Table 2. Production of HLA-B27 and hβ2m Transgenic Rats

			Founder			
			Integration[b]		Expression[c]	
Strain	Eggs[a]	Pups	B27	hβ2m	B27	hβ2m
LEW	348	23	8	7	5	4
F344	329	24	4	4	1	1

[a] Number of eggs injected and transferred to pseudopregnant recipients.

[b] Transgenic animals were identified by dot-blot analysis of DNA isolated from tails.

[c] Cell surface expression was assessed by indirect immunofluorescence and flow cytometry of PBLs.

and 2B), and in both lines the expression of the endogenous RT1 class I molecules appeared to be reduced in comparison with the nontransgenic control (Figure 2C).

Immunologic Function of the HLA-B27 Transgene

To assess T cell recognition of the B27 transgene product as a class I MHC antigen, primary grafts of B27 transgenic LEW rat skin were placed on nontransgenic LEW rats, and spleen cells from the recipient rats were subsequently tested for B27-specific cytotoxicity. As shown in Table 4,

Table 3. Copy Number and Cell Surface Expression of HLA-B27 and hβ2m in Transgenic Rat Lines

	Gene (Copy/Cell)[a]		Cell Surface Expression (Relative MCF)[b]	
Line	B27	hβ2m	B27	hβ2m
21-2	1	1	0.09	0.06
21-3	20	15	0.30	0.29
21-4L	6	6	0.74	0.42
21-4H	150	90	0.51	0.42
25-1	1	0	0.15	0.00
25-6	7	7	0.42	0.27
33-3	55	66	1.00	0.76

[a] Gene copy number was estimated by quantitative dot hybridization on DNA isolated from tails using probes specific for each transgene (see Figure 1A).

[b] Mean channel fluorescence (MCF) with antibodies to HLA-B (B1.23.2) or hβ2m (BBM.1) of PBLs from transgenic rats, relative to simultaneously determined MCF of PBLs from the B27/hβ2m transgenic mouse line 56-3. All data are from progeny of founders to eliminate influence of mosaicism.

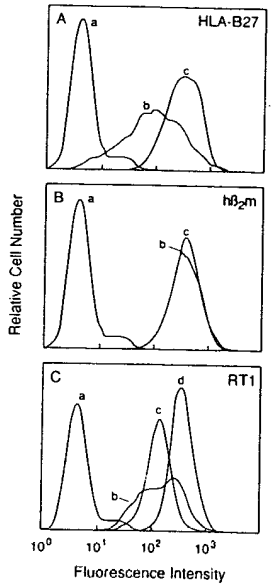


Figure 2. Comparison of Cell Surface Expression of HLA-B27, hβ2m, and the Endogenous RT1 Class I MHC Molecules in 21-4H, 21-4L, and Nontransgenic Rats

Peripheral blood mononuclear cells were incubated with saturating concentrations of monoclonal antibodies and fluorescein-labeled second antibodies and then analyzed by flow cytometry, as described in Experimental Procedures. The results demonstrate that cell surface expression of both transgenes was at least as high in the clinically normal 21-4L line as in the disease-prone 21-4H line and that endogenous RT1 expression appeared lower in the transgenic rats than in the nontransgenic control. Sources of cell populations were nontransgenic LEW stained with negative control antibody (a), 21-4H (b), 21-4L (c), and nontransgenic LEW stained with anti-RT1 antibody (d). Monoclonal antibodies were anti-HLA-B27 (B.1.23.2) (A), anti-hβ2m (BBM.1) (B), and anti-RT1 class I (OX18) (C).

Table 4. Cell-Mediated Cytotoxicity against HLA-B27

Effector Cells		Effector to	% Cytoxicity of Target Cells	
Donor	Recipient	Target Ratio	$B27^+h\beta_2m^+$	$B27^-h\beta_2m^+$
Experiment 1				
21-4L	LEW	100	34	15
		50	38	8
		20	36	3
LEW	LEW	100	19	13
		50	14	7
		20	5	1
Experiment 2				
21-4H	LEW	100	18	7
		50	8	4
		20	3	2
LEW	LEW	100	3	3
		50	1	2
		20	0	1

Spleen cells from LEW rats grafted 7 days earlier with skin from either 21-4 transgenic or normal LEW donors were incubated at the indicated effector target ratios with ^{51}Cr-labeled murine L cell targets expressing either $h\beta_2m$ alone or $h\beta_2m$ and HLA-B27. Incubation times: experiment 1, 6 hr; experiment 2, 4 hr. SD ≤15% in experiment 1 and <10% in experiment 2.

spleen cells from nontransgenic LEW rats receiving grafts from either 21-4H or 21-4L donors showed significantly higher lytic activity against L cell targets transfected with the B27 gene than against otherwise identical targets lacking this gene. Lytic activity was also higher in recipients of transgenic grafts than in recipients of control nontransgenic syngeneic grafts. These results indicate that the B27 transgene product is recognized in a conventional manner by allogeneically primed cytolytic T cells.

Inflammatory Disease in the 21-4H Line: Clinical and Histologic Findings

Gastrointestinal Tract

Overt disease appeared in all of the rats bearing the 21-4H transgene locus that survived past 10 weeks of age. This cohort consisted of 14 males and 9 females. The most common and persistent finding was diarrhea, manifested by frequent, voluminous, often watery stools. Diarrhea was observed in all 23 animals, with equal persistence and severity in the two sexes. Histologically, the gastrointestinal disease was manifested by chronic inflammation involving the stomach and small and large intestine (Figure 3). The distribution and severity of the lesions varied, the colon being the most consistently and prominently affected site. Less frequently, gastric lesions predominated. In all sites, the inflammatory cells consisted primarily of large and small lymphocytes, plasma cells, and smaller numbers of eosinophils. Although the inflammatory response remained primarily in the lamina propria, in the most severely affected regions it extended into the submucosa. Lymphocytes were commonly aggregated into small hyperplastic lymphoid foci, especially in the colon and ileum.

In the intestinal lesions, hyperplasia of crypt epithelial cells replaced mucus-secreting cells and increased the depth of the crypts (Figures 3D and 3F). Hyperplastic crypt cells showed regenerative atypia and a marked increase in mitotic activity. Destruction of crypts and/or the formation of crypt abscesses was uncommon and seen only in the most inflamed areas.

The gastric lesions generally consisted of widely scattered inflammatory foci in the lamina propria and submucosa, but in more severe lesions inflammation was much more extensive, and inflammatory cells accumulated in ectatic glands. The proliferation of mucus-neck cells resulted in marked reduction in the number of parietal cells (Figure 3B).

That the gastrointestinal inflammation did not result from a contagious pathogen was suggested by four pieces of evidence. Stool cultures for aerobic bacteria yielded only normal fecal flora. Furthermore, rats of the 21-4L line and nontransgenic LEW rats were housed for long periods in the same cages with affected 21-4H rats without showing any diarrhea or other signs of illness. In addition, the histology of the gastrointestinal tract of the affected 21-4H rats was not consistent with any known infectious process. Finally, diarrhea has also appeared in six out of seven transgenic rats of the 33-3 line past the age of 2 months, and not in their nontransgenic littermates.

Peripheral and Axial Joints

Peripheral arthritis was observed in 10 of 14 21-4H males and in 1 of 9 21-4H females. This was manifested in most cases by swelling, erythema, and tenderness of the tarsal joints of one or both hindlimbs (Figure 4B). In a few animals the carpal joints or digits were also inflamed (Figure 4D). The arthritis persisted from a few days to several weeks, and in some cases showed an undulating pattern of remission and exacerbation.

Histologically, large accumulations of neutrophils were present in the joint space. The synovium was hyperplastic, edematous and infiltrated with large numbers of lymphocytes, plasma cells, and neutrophils, with neutrophils predominating in the most active lesions (Figure 6B). There was marked pannus formation that eroded the bone at the synovial recess, invading and destroying the articular cartilage. Where the articular cartilage on adjacent joint surfaces was completely replaced by pannus, fibrous ankylosis occurred. Reactive bone formed small osteophytes along the diaphyses, and foci of metaplastic bone were seen within the fibrotic joint capsule. Chronic inflammation extended from the joint capsule to involve adjacent ligaments and tendons. Despite extensive joint destruction evident histologically, resolution generally occurred with preservation of mobility in the large joints.

Vertebral joints from two tails of 21-4H rats were examined histologically, and both revealed inflammatory changes at the outer aspects of the annulus fibrosus and its attachment to the vertebral endplate (Figure 6D). The inflammatory cells consisted of lymphocytes and small numbers of plasma cells mixed with active fibroblasts. There was active bone resorption at the insertion of the annulus and the adjacent periosteum was reactive.

Skin and Nails

Several animals of both sexes developed grossly evident

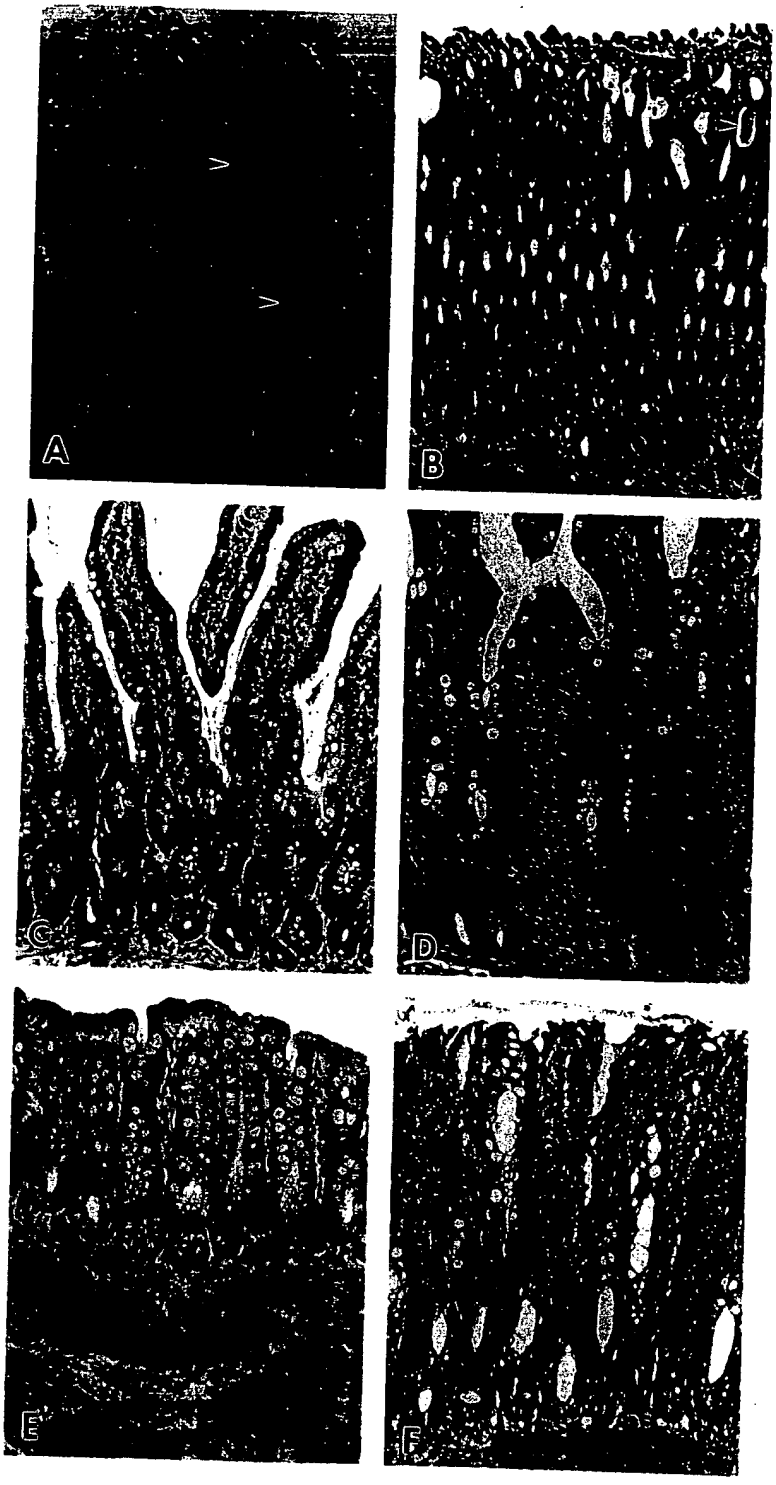


Figure 3. Gastrointestinal Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal stomach. Arrowheads indicate typical parietal cells (81.25×).
(B) Stomach of a 3-month-old 21-4H male, showing chronic gastritis, with numerous dilated pits and glands (asterisks). A microabscess is present in one dilated gland (arrowhead). Hyperplasia of the mucus-neck cells has largely replaced the parietal cells, and an inflammatory infiltrate is present throughout the lamina propria (65×).
(C) Normal ileum (84.5×).
(D) Ileum of a 3-month-old 21-4H male, showing chronic enteritis. The depth of the crypts is increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (84.5×).
(E) Normal colon (97.5×).
(F) Colon of a 3-month-old 21-4H male, showing chronic colitis. The depth of the crypts is markedly increased due to epithelial cell hyperplasia. There is a loss of mucus-secreting cells, and an inflammatory infiltrate is present throughout the lamina propria (97.5×).

changes in the tail skin and/or dramatic hyperkeratosis and dystrophy of the nails on all four extremities (Figures 5B and 5D). Histologically, in the tail lesions the epidermis was massively thickened by psoriasiform hyperplasia (Figure 6F). The rete ridges were regular and thickened at the base. Exocytosis of lymphocytes and neutrophils was common, with these cells accumulating in spongiotic foci in the epidermis, in the superficial parakeratotic crust, or around degenerated, necrotic keratinocytes. Diffuse orthokeratotic hyperkeratosis was prominent. The superfi-

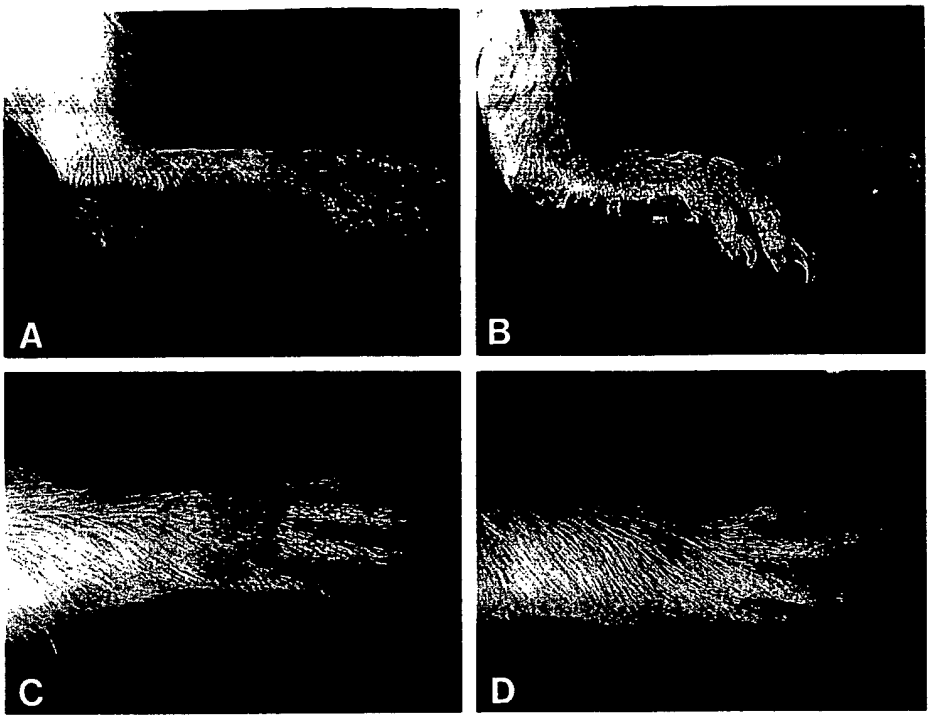


Figure 4. Peripheral Joint Gross Pathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal distal hindlimb.
(B) Distal hindlimb of a 6-month-old 21-4H male showing swelling and erythema.
(C) Normal distal forelimb.
(D) Distal forelimb of a 4-month-old 21-4H male showing swelling and erythema surrounding the carpal joint.

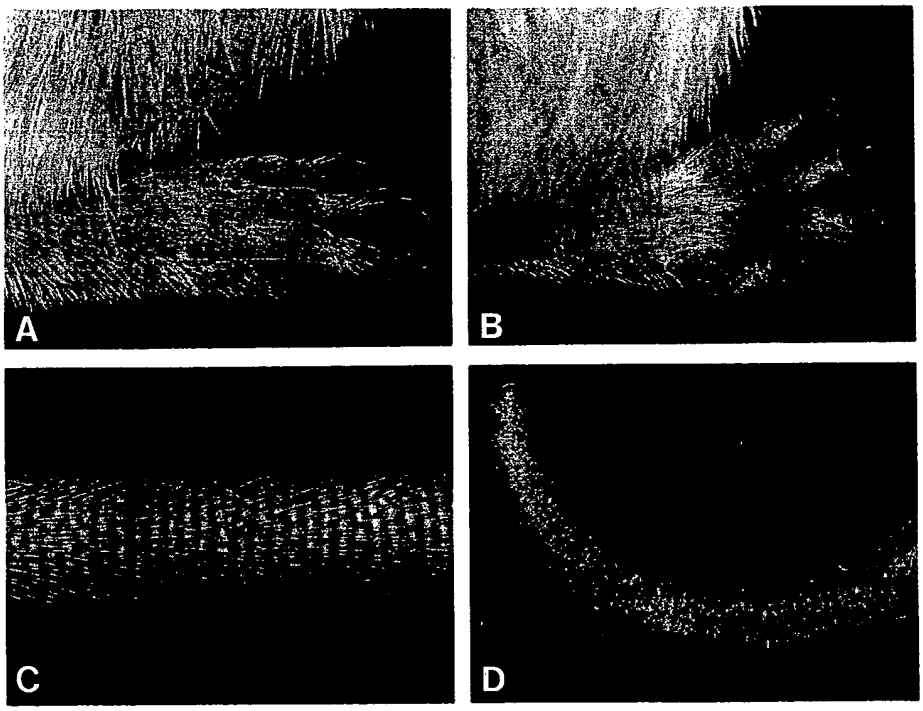


Figure 5. Nail and Skin Gross Pathology of 21-4H Rats

(A) Normal hindlimb digits and nails.
(B) Hindlimb digits and nails of a 3½-month-old 21-4 male, showing hyperkeratosis and dystrophy of the nails and alopecia over the digits.
(C) Normal tail.
(D) Tail of a 3½-month-old 21-4 male (same as in [B]), showing edema, alopecia, flaking, and masking of the normal ridged pattern.

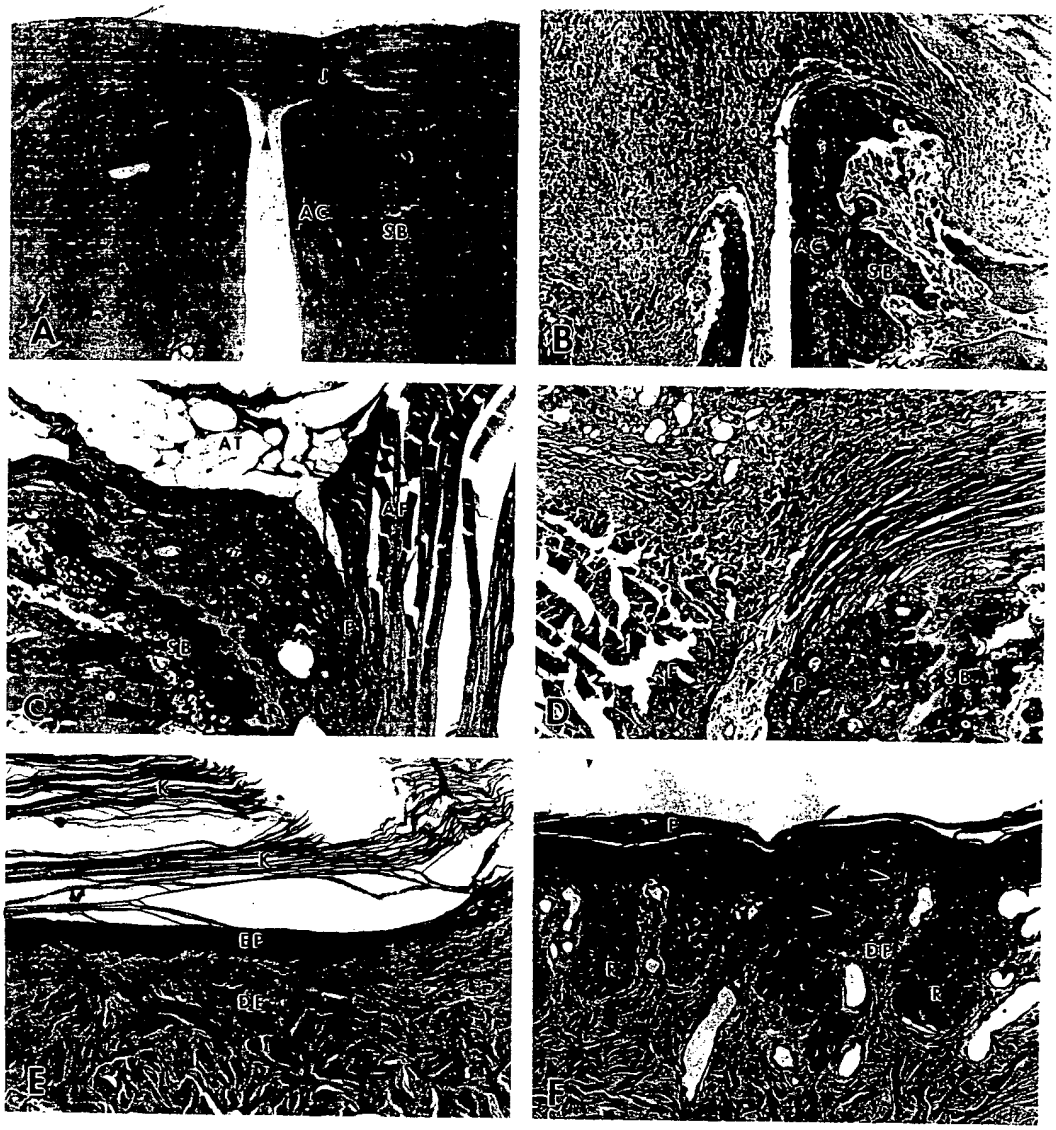


Figure 6. Peripheral and Axial Joint and Skin Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.
(A) Normal tarsal joint. Synovium (arrowhead), articular cartilage (AC), subchondral bone (SB), and joint capsule (J) are labeled (78×).
(B) Tarsal joint of a 4-month-old 21-4H male (same as in Figure 4D), showing chronic arthritis. There is a marked inflammatory infiltrate in the joint capsule and synovium, with pannus (asterisks) eroding articular cartilage (AC) and subchondral bone (SB) on both sides of the joint (58.5×).
(C) Normal tail intervertebral joint. The annulus fibrosus (AF), vertebral end plate (P), ossification center of subchondral bone (SB), and periarticular adipose tissue (AT) are labeled (65×).
(D) Tail intervertebral joint of a 4-month-old 21-4H male (same as in Figure 4D), oriented as a mirror image of (C), showing expansion of the periarticular connective tissue by mononuclear inflammation and fibrosis (asterisks), invading and disrupting the attachment of the outer layers of the annulus to the vertebral end plate (arrowheads). Annulus fibrosus, vertebral endplate, and subchondral bone are labeled as in (C) (58.5×).
(E) Normal tail skin. The keratin layer (K) overlies the epidermis (EP) and dermis (DE) (97.5×).
(F) Tail skin of a 3½-month-old 21-4 male (same as in Figure 5B), showing prominent, elongate, regular rete pegs (R) (psoriasiform epidermal hyperplasia), exocytosis of lymphocytes and neutrophils (arrowheads), parakeratosis (P), and dermal papillae (DP) containing inflammatory infiltrates (78×).

cial papillary dermis contained a diffuse infiltrate of neutrophils, lymphocytes, and plasma cells. Similar changes were seen in skin over the distal aspect of the digits.

Testis and Epididymis

Orchitis and epididymitis were prominent findings in the 21-4H males. The orchitis was manifested clinically by a progressive enlargement of the testes followed by testicular atrophy, with infertility ensuing by 3 months of age in most of the males. In contrast, the females showed little loss of fertility, even in the presence of persistent diarrhea. Histologically, the testicular tunica was thickened by connective tissue, which contained active angioblasts and fi-

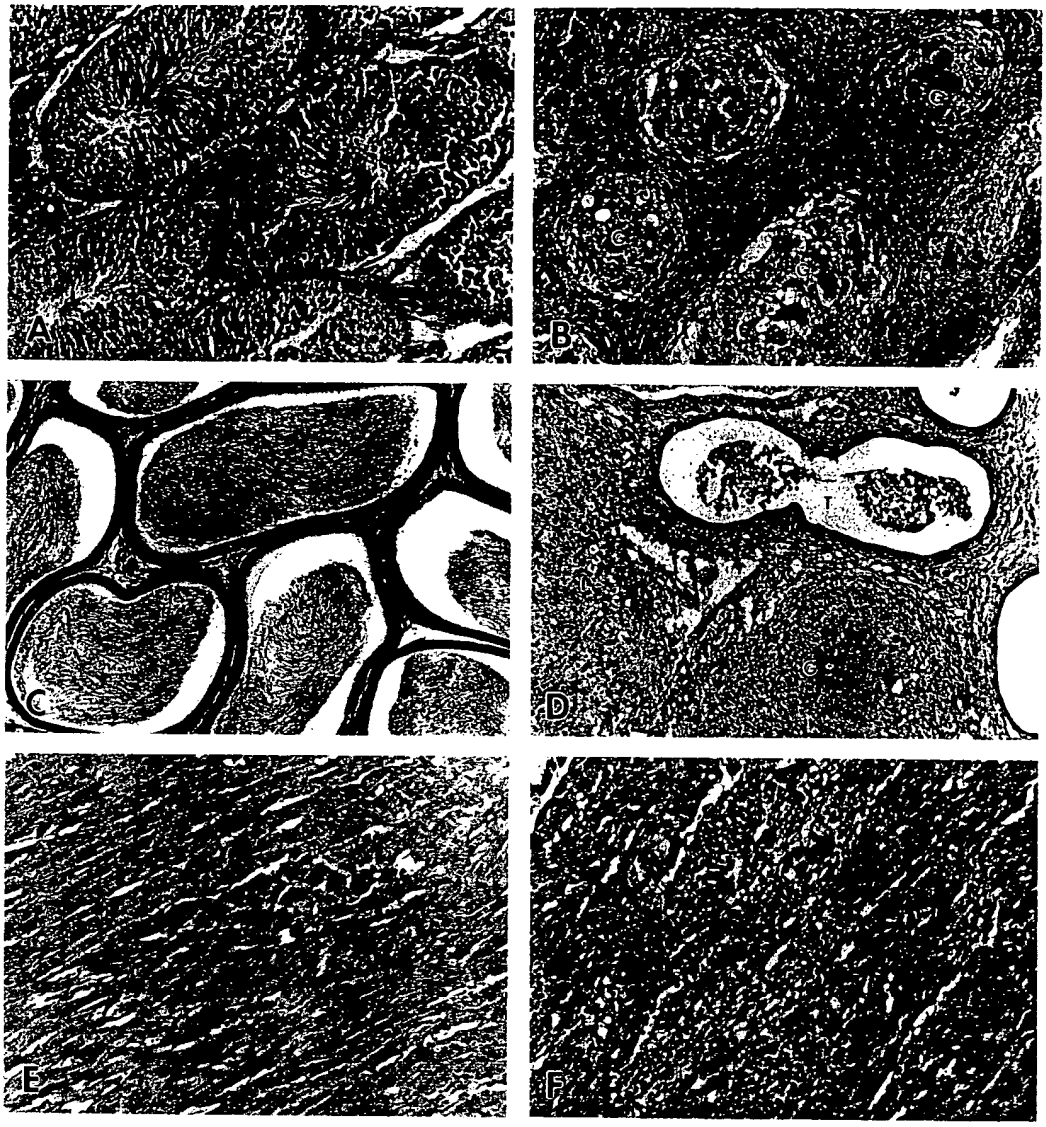


Figure 7. Male Genital Tract and Myocardial Histopathology of 21-4H Rats

Normal control specimens are from nontransgenic LEW rats, 3–6 months old.

(A) Normal testis (71.5×).

(B) Testis of a 3-month-old 21-4H male, showing chronic orchitis with an intense mononuclear cell interstitial inflammatory infiltrate and sperm granulomas (G) (71.5×).

(C) Normal epididymis (65×).

(D) Epididymis of a 3-month-old 21-4H male, showing chronic epididymitis with a granulomatous interstitial inflammatory cell infiltrate (I), sperm granuloma (G), and dilated tubules (T) containing degenerated inflammatory cells and no sperm (65×).

(E) Normal myocardium (78×).

(F) Myocardium of a 3-month-old 21-4H male (same as in Figure 3F), showing myocarditis with a prominent mononuclear inflammatory cell infiltrate separating the myofibers (78×).

broblasts as well as large numbers of lymphocytes and plasma cells. The testes often contained numerous granulomas with necrotic centers surrounded by epithelioid macrophages and giant cells and peripherally by lymphocytes, plasma cells, and fibrosis (Figure 7B). Central infarction of the testis was a common finding in the most severely affected specimens.

The epididymis frequently contained granulomas similar to those found in the testis, along with dilated tubules containing necrotic cellular debris. The interstitium of the epididymis was expanded by lymphocytes, plasma cells, epithelioid macrophages, and moderate fibrosis (Figure 7D).

Heart

Active inflammatory lesions were evident histologically in four of nine 21-4H hearts examined (Figure 7F). In one specimen, extensive multifocal lesions were seen, involving the ventricular walls and septum. The lesions con-

sisted of large numbers of lymphocytes and small numbers of plasma cells, macrophages, and eosinophils. The myofibers were widely separated by the inflammatory cells, and scattered karyorrhectic nuclei were seen. In the less severely affected specimens, infiltrates of lymphocytes and plasma cells were found at the root of the aortic valve. In more chronic lesions there was moderate fibrosis scattered throughout the myocardium accompanied by mild lymphocytic inflammation. In one animal the adventitia of the great vessels was infiltrated by large numbers of lymphocytes and plasma cells admixed with proliferating angioblasts and fibroblasts.

Eye and Central Nervous System

Mild keratitis and anterior uveitis were observed histologically in one of five eyes from 21-4H rats, one of five eyes from 21-4L rats, and none of four eyes from nontransgenic LEW rats. These findings were judged to be nonspecific, probably secondary to bacterial keratitis.

A peculiar neurologic syndrome was seen in all of the females and most of the males of the 21-4H line. This was manifested by cerebellar ataxia, with intermittent episodes of a stereotypical muscular dystonia, usually in response to handling or some other mild stimulus. Electrophysiologic studies during these episodes demonstrated increased muscular tone without evidence of a cortical seizure focus (data not shown). For several reasons, this abnormality was thought to result from a process distinct from that giving rise to the other lesions. Whereas the other lesions appeared after puberty and then progressed, the neurologic abnormality began within a few weeks after birth and showed no increase in severity thereafter. Unlike the other disease processes, the clinical pattern of the neurologic findings showed little variation from rat to rat. Furthermore, the histologic abnormalities associated with the neurologic disease, which involved primarily the spinal cord and cerebellum, were not inflammatory (data not shown). Finally, there was no evidence of neurologic disturbance in the transgenic F344 line that also showed diarrhea, nor in any of the other transgenic LEW lines.

Other Tissues

The following tissues were examined in at least one of the 21-4H rats showing diarrhea and found not to show histologic abnormalities: esophagus, lung, liver, kidney, adrenal, pancreas, penis, spleen, and thymus. Atrophy of thymus and spleen that was apparent to gross examination was a common finding, however, along with peripheral and mesenteric lymph node enlargement.

Clinical and Histologic Findings in Other Transgenic Lines

No clinical abnormalities were noted in any of the B27 transgenic LEW lines other than 21-4H. Histologic tissue surveys of several 21-4L rats revealed a mild degree of intestinal lymphoid hyperplasia and fibrosis as the only abnormality. Similar intestinal lesions were also found at a lower frequency in nontransgenic controls, and hence the significance of these findings in the 21-4L rats is not yet established. As noted above, almost all transgenic rats of the F344 line 33-3 showed diarrhea by 2 months of age.

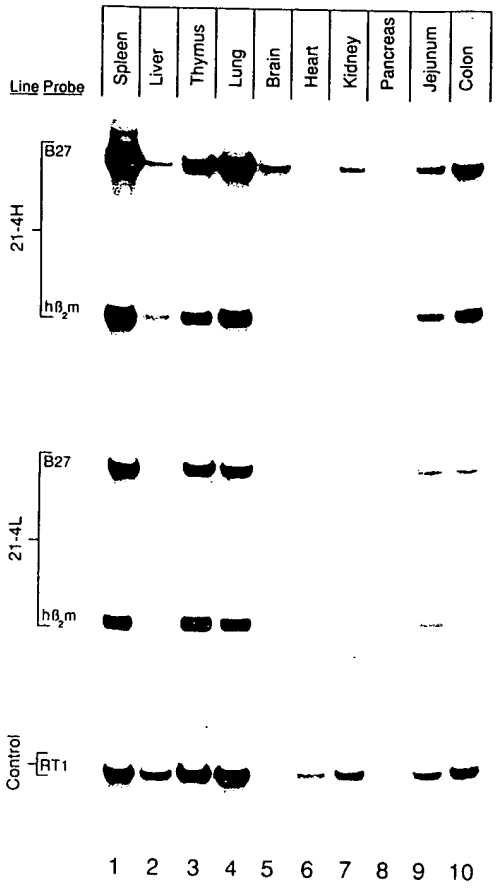


Figure 8. Northern Blot Analysis of HLA-B27, hβ2m, and RT1 mRNA: Tissue Survey

Total cellular RNA from tissues of 12-week-old male 21-4H, 21-4L, and nontransgenic control rats was subjected to denaturing agarose gel electrophoresis (10 μg per lane), transferred to nylon membranes, and hybridized to ^{32}P-labeled probes as described in Experimental Procedures and Figure 1. Membranes were exposed to XAR-5 film at −70°C with intensifying screens for 2–26 hr.

Tissue Distribution of mRNA Expression

Despite the striking differences in disease manifestations, the 21-4H and 21-4L lines showed similar cell surface expression of the transgene products in PBLs (Table 3; Figures 2A and 2B). It was thus of interest to compare the two lines with respect to the level and tissue distribution of mRNA transcripts of both transgenes. Northern blot analysis was carried out on total cellular RNA isolated from tissues of a limited number of rats of the 21-4H and 21-4L lines. HLA-B27 mRNA was detected with a 350 bp probe from the HLA-B 3′ untranslated region (probe C in Figure 1A), and hβ2m mRNA was detected with the same probe used to detect hβ2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp probe from the 3′ untranslated region of the RT1.A gene. Figures 8 and 9 contain results from age- and sex-matched representatives of the 21-4H and 21- 4L lines and a nontransgenic control.

As shown in Figure 8, the distribution and relative abundance of both B27 and hβ2m transgene transcripts among

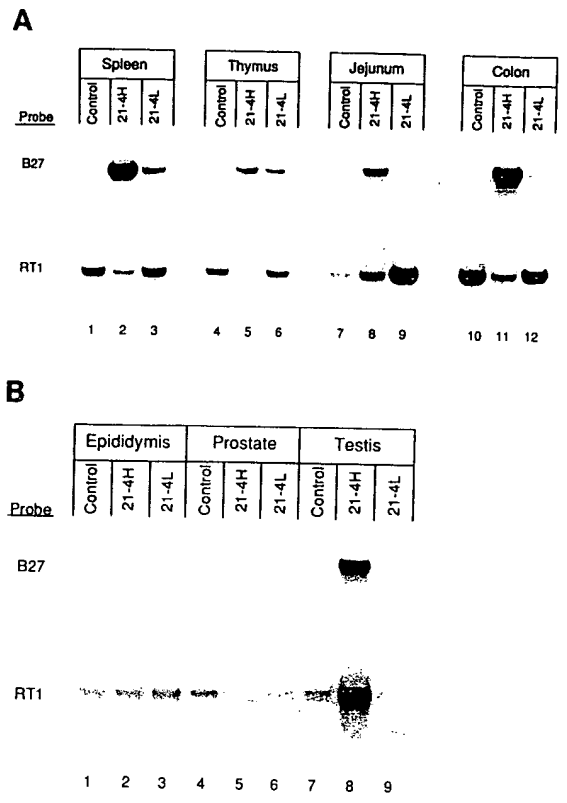


Figure 9. Northern Blot Analysis of HLA-B27 and RT1 mRNA: Comparative Analysis of Seven Tissues

(A) Tissue sources and methods were the same as described in Figure 8. Membranes were exposed 1–8 hr.

(B) Tissue sources were the same as described in Figure 8. Five micrograms of total cellular RNA was added per lane. Prostatic tissue in the 21-4H animal was difficult to identify because of severe atrophy, presumed to be due to loss of androgen stimulation. The membrane probed for B27 was exposed for 1 hr. A 10 hr exposure showed B27 transcripts in 21-4H epididymis (data not shown). The membrane probed for RT1 was exposed for 5 hr.

the various tissues examined were similar to those of the endogenous RT1 class I expression and typical of MHC class I gene expression (Klein, 1986). In addition, both transgenes produced mRNA transcripts of the predicted size.

Figure 9 shows direct comparisons of the 21-4H and 21-4L lines with respect to the relative amounts of B27 and RT1 transcripts in tissues affected by the disease process in the 21-4H line. The abundance of B27 transcripts was dramatically higher in the 21-4H rat than in the 21-4L rat in spleen, colon, and testis, and less markedly increased in jejunum and epididymis. In the thymus, the B27 transcripts were approximately equal in the two lines; however, this may have been a reflection of thymic atrophy in the 21-4H rats.

Although the apparent reduction of RT1 cell surface expression in PBLs was comparable in 21-4H and 21-4L rats (Figure 2C), at the level of mRNA there was no apparent reduction of RT1 transcripts in the 21-4L tissues examined. In contrast, the abundance of RT1 transcripts was markedly reduced in 21-4H spleen, thymus, and colon, compared with tissues from a nontransgenic rat. High expression of RT1 mRNA was found in the 21-4H testis and jejunum. In the case of testis, this probably reflects the intense infiltration of inflammatory cells seen histologically in this organ (Figure 7B), whereas an explanation for the finding in jejunum is less apparent.

Discussion

Integration and Expression of HLA-B27 and $h\beta_2m$ Transgenes in Rats

In an attempt to create an animal model of B27-associated disease, we developed transgenic technology in rats and produced inbred rats expressing both HLA-B27 and $h\beta_2m$. Simultaneously, Mullins et al. (1990), using similar methods, were independently successful in producing transgenic rats expressing a mouse renin gene.

The levels of B27 and $h\beta_2m$ mRNA transcripts in the transgenic tissues paralleled those of the endogenous class I genes in nontransgenic tissues, suggesting that the transgenes were subject to physiologic regulation. It is interesting that the presence of the human transgenes resulted in an apparently reduced expression of the endogenous class I RT1 genes, at the level of cell surface protein expression in PBLs and/or at the level of mRNA, both in lymphoid and nonlymphoid tissue. The possibility was not excluded that the reduced binding of OX18 antibody to the transgenic PBLs was due to an effect of $h\beta_2m$ either on the number of cell surface RT1 class I molecules or on the affinity of the OX18 antibody for these molecules. However, such an effect would not explain the prominent reduction in RT1 mRNA transcripts seen in the 21-4H spleen, thymus, and colon.

Several transcriptional regulatory elements have been identified in the 200 bp 5′ to the transcription initiation site in murine class I MHC genes, including the binding site for the conserved nuclear factor KBF1 (David-Watine et al., 1990; Kieran et al., 1990), and homologous sequences are found in the HLA-B27 promoter region (Weiss et al., 1985). Thus, at least part of the inhibition of RT1 transcription in the 21-4H tissues might be explained by competition by the transgenes for nuclear factor binding.

The Inflammatory Disease of the 21-4H Transgenic Rats: Comparison with B27-Associated Disease in Humans

B27-associated disorders in humans encompass a spectrum of inflammatory diseases affecting predominantly the peripheral and axial musculoskeletal system, gastrointestinal tract, genital tract, integument, and eye (Table 1). Less common involvement of heart and nervous system and rare involvement of lung are also observed in these disorders (Bulkley and Roberts, 1973; Good, 1974; Taurog and Lipsky, 1990). The spontaneously arising disease in B27/$h\beta_2m$ transgenic rats showed a striking clinical and histologic similarity to B27-associated disease in humans, with inflammatory lesions of peripheral and axial joints, gut, male genital tract, nails, skin, and heart. The close resemblance of the findings in the transgenic rats

to B27-associated disease in humans strongly supports the conclusion that the B27 molecule itself participates in the pathogenesis of the various lesions found in different organ systems in the spondyloarthropathies.

The most prevalent site of inflammation in the transgenic rats appears to be the gastrointestinal tract. All of the 21-4H rats under observation for at least 6 months developed overt diarrhea, and a similar picture is emerging in the 33-3 line. These findings suggest that the events initiating the disease process occur in the gastrointestinal tract and that further investigation of the intestinal immunophysiology and immunopathology of the transgenic animals may provide some insight into the role of the B27 molecule in these events.

Numerous observations in humans support a causal link between factors in the gut and inflammatory joint disease. Peripheral and axial arthritis are common accompaniments of chronic inflammatory bowel disease even in the absence of B27 (Table 1), and recent evidence suggests that milder degrees of gastrointestinal inflammation are closely correlated with the occurrence of B27-associated joint disease in individuals without bowel symptoms. Histologic examination of endoscopically obtained biopsies in a large series of patients with reactive arthritis or ankylosing spondylitis indicated that over 60% had asymptomatic inflammatory lesions of the terminal ileum or colon (Cuvelier et al., 1987). Whether patients with B27-associated disease develop inflammatory lesions in the more proximal small intestine or stomach that might resemble those seen in the 21-4H rats is not known.

Although gastrointestinal inflammation in the transgenic rats was present equally in both sexes, arthritis occurred predominantly in males. This closely followed the pattern in humans, in whom males with ankylosing spondylitis, juvenile onset spondyloarthropathy, or reactive arthritis following genital infection outnumber females 3- to 10-fold. The prevalence of subclinical gastrointestinal inflammation in B27 individuals without rheumatic disease, either male or female, is not known. Both peripheral and axial arthritis occurred in the 21-4H rats. Clinically, the peripheral arthritis resembled that seen in other experimental models of arthritis in rats, such as those induced by complete Freund's adjuvant or streptococcal cell walls, with swelling and erythema of the proximal hind paw being the predominant lesion. Histologically, the involved joints showed lesions typical of experimental arthritis in rats, as well as B27-associated peripheral arthritis in humans, with synovial hyperplasia, inflammatory cell infiltration, pannus formation, and destruction of articular cartilage and bone (Greenwald and Diamond, 1988; Taurog et al., 1988b).

Axial arthritis, with inflammatory cell infiltration and periosteal reaction at the margins of the intervertebral discs, was seen histologically in the tails of 21-4H rats. This appears to be the same pathologic process that leads to the vertebral changes in ankylosing spondylitis, although histologic comparison of this lesion with human spondylitis is made difficult by the paucity of descriptions of early lesions in humans (Ball, 1971; Eulderink, 1990). More generally, the vertebral lesion in the 21-4H rats also closely resembles the enthesitis, inflammation at ligamentous attachments to bone, that is a pathologic hallmark of the B27-associated diseases in humans (Ball, 1971).

Dramatic psoriasiform skin and nail lesions developed in the 21-4H rats. These lesions show an extraordinary histologic resemblance to psoriatic lesions in humans. Although in most patients with psoriasis vulgaris there is no association with HLA-B27, lesions termed keratoderma blenorrhagica that are histologically indistinguishable from the psoriatic variant pustular psoriasis are commonly found in B27-associated reactive arthritis (Good, 1974; Keat, 1983). Furthermore, typical psoriasis vulgaris occasionally supervenes in patients initially presenting with reactive arthritis. Finally, a common pathogenetic mechanism between psoriasis vulgaris and B27-associated disease is suggested by the recent observation that both psoriasis vulgaris and the skin lesions of Reiter's syndrome appear to be significantly exacerbated in patients with coexistent infection with the human immunodeficiency virus HIV-1 (Duvic et al., 1987).

Another striking lesion in the 21-4H rats was orchitis, which was found in virtually all of the males, invariably in association with epididymitis. In humans, urogenital inflammation is prevalent in B27-associated diseases. Although urethritis in males with reactive arthritis is a common finding even in the absence of known urethral infection, prostatitis and epididymitis in males, cervicitis in females, and cystitis in both sexes have been described (Yli-Kerttula, 1984). Although there have been no reports of histologically confirmed orchitis associated with HLA-B27 or with B27-associated syndromes, clinical descriptions suggestive of orchitis have been published (Montanaro and Bennett, 1984). It is thus not altogether unlikely that the inflammatory process induced by B27 in the 21-4H rat testis has a milder human counterpart.

Inflammatory disease involving the root of the aortic valve and myocardium was found in the 21-4H rats. Both aortic insufficiency and cardiac conduction disturbances are well-documented complications of ankylosing spondylitis and reactive arthritis (Bergfeldt et al., 1988; Bulkley and Roberts, 1973; Good, 1974). Moreover, primary myocardial disease may also be relatively prevalent in ankylosing spondylitis (Brewerton et al., 1987). The cardiac pathology of the 21-4H rats, like the lesions in the peripheral and axial joints, gastrointestinal tract, skin, and male genital tract, thus appears to be a direct counterpart of a pathologic process in B27-associated human disease.

In comparing the pathologic lesions identified in the B27 transgenic rats with B27-associated disease in humans, only the neurologic disease in the 21-4H LEW line seemed to represent a significant anomaly. Occasional cases of either central or peripheral neurologic disease have been reported in association with B27-associated reactive arthritis (Good, 1974; Montanaro and Bennett, 1984; Taurog and Moore, 1986), but none of these has been characterized histologically, nor do their clinical descriptions resemble the findings in the 21-4H rats. As mentioned under Results, the neurologic lesions in the 21-4H rats appear to be temporally and histologically unrelated to the inflammatory disease seen in other organs.

Although the possibility cannot be excluded that the neurologic disturbance contributed indirectly to the inflammatory lesions, for example by disruption of the normal innervation of lymphoid tissue or gut (Anderson, 1990), the absence of neurologic disease in the 33-3 line, a second transgenic line exhibiting spontaneously occurring B27-associated disease, suggests that the neurologic disease in the 21-4H line is not a necessary part of the inflammatory process in other organ systems, but likely a result of a dominant insertional mutation. A complete description of the neurologic findings in the 21-4H line is in progress.

The Inflammatory Disease of B27/hβ_2m Transgenic Rats: Possible Mechanisms

It is unclear why overt inflammatory disease developed in only two of the seven transgenic rat lines, 21-4H and 33-3. It is unlikely that differences in postconceptional environment play a significant role in determining the phenotypes of the different transgenic lines, since segregation of the diseased phenotype with the 21-4H locus was uniformly observed in litters containing both 21-4H and 21-4L offspring. Insertional mutation appears unlikely as an explanation, since two independent transgenic lines developed aspects of a similar disease. Nor was evidence obtained for differences in B27 function, since the 21-4H and 21-4L lines comparably stimulated immune recognition of B27 by cytolytic T cells. The variation among transgenic rat lines most likely can be ascribed to either quantitative or qualitative differences in the expression of the transgenes or to differing effects of the transgene on the host genome.

The results presented in this study do not exclude the possibility that a human class I MHC gene other than HLA-B27 might also be capable of producing a disease process similar to that described here, nor do they exclude the possibility that the hβ_2m gene alone might be sufficient to produce disease. Studies are in progress to address these possibilities.

Several lines of evidence have suggested that interactions between B27 and bacterial products are involved in the pathogenesis of the spondyloarthropathies (Yu et al., 1989). Although the disease in the transgenic rats arose spontaneously in the apparent absence of infection by pathogens, the possibility must be considered that the pathogenesis involves interactions between B27 and commensal organisms such as the intestinal flora or pathogens not detected by routine serologic screening. Studies in which the transgenic rats are maintained germ free will be important in exploring this issue.

Despite extensive investigation of the structure and function of class I MHC genes in general and HLA-B27 in particular, it has so far not been possible to identify the molecular mechanism of the association of B27 with human disease. However, given the close resemblance of the spontaneous disease of the 21-4H line to B27-associated human disorders, a detailed cellular and molecular analysis of the B27/hβ_2m transgenic rats should enhance our understanding of the role of HLA-B27 in causing disease. It may also contribute to a broader understanding of the function of class I MHC molecules.

Experimental Procedures

Animals

Specific pathogen-free inbred Lewis/CrlBR (LEW) and Fischer F-344/CrlBR (F344) rats, and outbred Sprague-Dawley rats, were purchased from Charles River Laboratories, Boston, MA. Hybrid mice of the transgenic line 56-3 (Taurog et al., 1990), which express high levels of both B27 and hβ_2m on lymphoid cell surfaces, were bred in our animal colony. Animals were maintained in accordance with institutional guidelines.

Generation and Identification of Transgenic Rats

Immature LEW or F344 female rats were superovulated according to the method of Armstrong and Opavsky (1988) and bred with fertile males. The day following breeding, fertilized one-cell eggs were flushed from the oviduct of females exhibiting either vaginal plugs or sperm in vaginal lavage fluid. Eggs were held in Brinster's medium for 2 hr or less before microinjection. Microinjection of eggs and transfer to day 1 pseudopregnant Sprague-Dawley females were carried out essentially as described for mice (Brinster et al., 1985).

Two genomic clones were used for microinjection of fertilized rat eggs (Figure 1). The HLA-B27 gene encoding the HLA-B* 2705 subtype (Bodmer et al., 1990) was contained on a 6.5 kb EcoRI fragment (clone pE.1-B27; Taurog et al., 1988a; Taurog and El-Zaatari, 1988) and the hβ_2m gene was contained on a 15 kb SalI–PvuI fragment (clone pβ2m-13, the gift of Dr. H. L. Ploegh, Amsterdam, The Netherlands; Güssow et al., 1987). Each insert was separated from plasmid DNA by agarose gel electrophoresis and isolated by perchlorate elution (Chen and Thomas, 1980). The solution used for microinjection contained both fragments, each at 1.5 ng/μl.

Identification and quantitation of transgenes were determined in the founder animals and their progeny by dot-blot hybridization of genomic DNA isolated from tail biopsies, as previously described (Brinster et al., 1985). Genomic DNA was analyzed by hybridization with 5′ and 3′ flanking probes for the *HLA-B* locus, as previously described (probes A and B in Figure 1A; Taurog et al., 1988a), and with a 3.7 kb BglII fragment containing exons 2 and 3 of the hβ_2m gene (probe D in Figure 1B).

RNA Analysis by Northern Blot Hybridization

Northern blot hybridization was carried out as described elsewhere (S. D. Maika, L. Laimonis, A. Messing, and R. E. Hammer, submitted). Briefly, total cellular RNA was extracted from tissues by the guanadinium isothiocyanate–CsCl procedure, separated on glyoxal agarose gels, and blotted onto nylon membranes. HLA-B27 mRNA was detected with the 350 bp HLA-B 3′ untranslated region probe pHLA-1.1 (probe C in Figure 1A; Koller et al., 1984), and hβ_2m mRNA was detected with the same 3.7 kb BglII fragment used to detect hβ_2m genomic DNA (probe D in Figure 1B). RT1 class I mRNA was detected with a 447 bp PvuII–HindIII fragment containing the 3′ untranslated region of the RT1.A^a gene pBS3.3/1 (the gift of Dr. J. C. Howard, Cambridge, England; Rada et al., 1990). All stringency washes were carried out in 0.1× SSC, 0.5% SDS at 65°C.

Monoclonal Antibodies, Indirect Immunofluorescence, and Flow Cytometry

The following murine monoclonal antibodies were used: B.1.23.2, IgG_{2b}, binding a monomorphic determinant shared by HLA-B and -C molecules (Rebai and Malissen, 1983); BBM.1, IgG_{2b}, binding hβ_2m (Brodsky et al., 1979); and OX18, IgG_1, binding a monomorphic rat RT1 class I antigen (Fukumoto et al., 1982). P1.17, an IgG_{2a} myeloma, served as a negative control.

Indirect immunofluorescence was carried out as previously described (Taurog and El-Zaatari, 1988; Taurog et al., 1988a). Briefly, Ficoll–Hypaque-purified peripheral blood mononuclear cells were incubated with saturating concentrations of each monoclonal antibody, washed, then incubated with fluorescein-conjugated F(ab)′$_2$ fragments of goat anti-mouse Fcγ antibodies (Cappel Inc., Malvern, PA). After washing, the cells were fixed in 1% paraformaldehyde before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Viable lymphocytes were analyzed by gating of forward and 90° light scatter.

Generation and Analysis of Cytolytic T Cells

Primary alloimmunization by skin grafting was carried out by the method of Peter and Feldman (1972). Seven days after graft placement, recipient spleen cells were used as effector cells in a 4–6 hr ^{51}Cr release assay, as previously described (Taurog et al., 1988a). Two mouse L cell lines were used as target cells, one transfected with and expressing the hβ2m gene, the other transfected with and expressing both the HLA-B*2705 and hβ2m genes, as previously described (El-Zaatari et al., 1990).

Histology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Joints were embedded and sectioned following fixation and decalcification for 4–6 weeks in 10% disodium EDTA, as previously described (Taurog et al., 1988b), or following decalcification in 10% formic acid. Eyes were embedded in methacrylate before sectioning and staining.

Acknowledgments

The technical contributions of Jana L. Hlavaty and Beverly Y. Hastings are gratefully acknowledged. The authors thank Dr. David T. Armstrong for providing data prior to publication; Dr. Jonathan C. Howard for providing the pBS3.3/1 gene and data prior to publication; Dr. Jerry Y. Niederkorn and Marsha S. Pidherney for assistance with histologic sections of eyes; Dr. Albee Messing and Dr. Gerald A. Marks for assistance with preliminary characterization of the neurologic disturbance in the 21-4H line; and Dr. Joseph L. Goldstein, Dr. Michael S. Brown, and Dr. Peter E. Lipsky for helpful discussions and critical review of the manuscript. R. E. H. and J. D. T. also wish to thank Dr. Lipsky for encouraging us to undertake this project. This work was supported by NIH grants AR09989 (J. D. T. and R. E. H.) and RR00890 (J. A. R.) and a grant from the North Texas Chapter of the Arthritis Foundation (J. D. T. and R. E. H.). J. D. T. was supported by NIH Research Career Development Award AR01756 during part of this work.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received October 8, 1990.

References

Anderson, A. O. (1990). Structure and organization of the lymphatic system. In Immunophysiology, J. J. Oppenheim and E. M. Shevach, eds. (New York: Oxford University Press), pp. 14–45.

Armstrong, D. T., and Opavsky, M. A. (1988). Superovulation of immature rats by continuous infusion of follicle-stimulating hormone. Biol. Reprod. *39*, 511–518.

Ball, J. (1971). Enthesopathy of rheumatoid and ankylosing spondylitis. Ann. Rheum. Dis. *30*, 213–223.

Bergfeldt, L., Insulander, P., Lindblom, D., Möller, E., and Edhag, O. (1988). HLA-B27: an important genetic risk factor for lone aortic regurgitation and severe conduction system abnormalities. Am. J. Med. *85*, 12–18.

Bodmer, J. G., Marsh, S. G. E., Parham, P., Erlich, H. A., Albert, E., Bodmer, W. F., Dupont, B., Mach, B., Mayr, W. R., Sasazuki, T., Schreuder, G. M. T., Strominger, J. L., Svejgaard, A., and Terasaki, P. I. (1990). Nomenclature for factors of the HLA system, 1989. Hum. Immunol. *28*, 326–342.

Brewerton, D. A., Hart, F. D., Caffrey, M., Nicholls, A., James, D. C. O., and Sturrock, R. D. (1973). Ankylosing spondylitis and HL-A 27. Lancet *1*, 904–907.

Brewerton, D. A., Gibson, D. G., Goddard, D. H., Jones, T. J., Moore, R. B., Pease, C. T., Revell, P. A., Shapiro, L. M., and Swettenham, K. V. (1987). The myocardium in ankylosing spondylitis. A clinical, echocardiographic, and histopathologic study. Lancet *1*, 995–998.

Brinster, R. L., Chen, H. Y., Trumbauer, M. E., Yagle, M. K., and Palmiter, R. D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. Proc. Natl. Acad. Sci. USA *82*, 4438–4442.

Brodsky, F. M., Bodmer, W. F., and Parham, P. (1979). Characterization of a monoclonal anti-β2-microglobulin antibody and its use in the genetic and biochemical analysis of major histocompatibility antigens. Eur. J. Immunol. *9*, 536–545.

Bulkley, B. H., and Roberts, W. C. (1973). Ankylosing spondylitis and aortic regurgitation. Description of the characteristic cardiovascular lesion from study of eight necropsy patients. Circulation *48*, 1014–1027.

Calin, A., ed. (1984). Spondylarthropathies (Orlando, Florida: Grune & Stratton).

Chen, C. W., and Thomas, C. A. J. (1980). Recovery of DNA segments from agarose gels. Anal. Biochem. *101*, 339–341.

Cuvelier, C., Barbatis, C., Mielants, H., De Vos, M., Roels, H., and Veys, E. (1987). Histopathology of intestinal inflammation related to reactive arthritis. Gut *28*, 394–401.

David-Watine, B., Israël, A., and Kourilsky, P. (1990). The regulation and expression of MHC class I genes. Immunol. Today *11*, 286–292.

Duvic, M., Johnson, T. M., Rapini, R. P., Freese, T., Brewton, G., and Rios, A. (1987). Acquired immunodeficiency syndrome—associated psoriasis and Reiter's syndrome. Arch. Dermatol. *123*, 1622–1632.

El-Zaatari, F., Sams, K. C., and Taurog, J. D. (1990). In vitro mutagenesis of HLA-B27. Amino acid substitutions at position 67 disrupt anti-B27 monoclonal antibody binding in direct relation to the size of the substituted side chain. J. Immunol. *144*, 1512–1517.

Eulderink, F. (1990). Pathology of ankylosing spondylitis. Spine: State of the Art Reviews *4*, 507–528.

Fukumoto, T., McMaster, W. R., and Williams, A. F. (1982). Mouse monoclonal antibodies against rat major histocompatibility antigens. Two Ia antigens and expression of Ia and class I antigens in rat thymus. Eur. J. Immunol. *12*, 237–243.

Good, A. E. (1974). Reiter's disease: a review with special attention to cardiovascular and neurologic sequelae. Semin. Arthritis Rheum. *3*, 253–286.

Greenwald, R. A., and Diamond, H. S., eds. (1988). Handbook of Animal Models for the Rheumatic Diseases, Vol. 1 (Boca Raton, Florida: CRC Press).

Güssow, D., Rein, R., Ginjaar, I., Hochstenbach, F., Seemann, G., Kottman, A., and Ploegh, H. L. (1987). The human β2-microglobulin gene. Primary structure and definition of the transcriptional unit. J. Immunol. *139*, 3132–3138.

Keat, A. (1983). Reiter's syndrome and reactive arthritis in perspective. N. Engl. J. Med. *309*, 1606–1615.

Khan, M. A., and van der Linden, S. M. (1990). Ankylosing spondylitis: clinical aspects. Spine: State of the Art Reviews *4*, 529–551.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., and Israël, A. (1990). The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell *62*, 1007–1018.

Kievits, F., Ivanyi, P., Krimpenfort, P., Berns, A., and Ploegh, H. L. (1987). HLA-restricted recognition of viral antigens in HLA transgenic mice. Nature *329*, 447–449.

Klein, J. (1986). Natural History of the Major Histocompatibility Complex (New York: John Wiley & Sons).

Koller, B. H., Sidwell, B., DeMars, R., and Orr, H. T. (1984). Isolation of HLA locus-specific DNA probes from the 3′-untranslated region. Proc. Natl. Acad. Sci. USA *81*, 5175–5178.

Krimpenfort, P., Rudenko, G., Hochstenbach, F., Güssow, D., Berns, A., and Ploegh, H. (1987). Crosses of two independently derived transgenic mice demonstrate functional complementation of the genes encoding heavy (HLA-B27) and light (β2-microglobulin) chains of HLA class I antigens. EMBO J. *6*, 1673–1676.

Moll, J. M., Haslock, I., Macrae, I. F., and Wright, V. (1974). Associations between ankylosing spondylitis, psoriatic arthritis, Reiter's disease, the intestinal arthropathies, and Behcet's syndrome. Medicine (Baltimore) *53*, 343–364.

Montanaro, A., and Bennett, R. M. (1984). Myelopathy in Reiter's disease. J. Rheumatol. *11*, 540–541.

Mullins, J. J., Peters, J., and Ganten, D. (1990). Fulminant hypertension in transgenic rats harbouring the mouse *Ren-2* gene. Nature *344*, 541–544.

Nickerson, C. L., Luthra, H. L., Savarirayan, S., and David, C. (1990). Susceptibility of HLA-B27 transgenic mice to Yersinia enterocolitica infection. Hum. Immunol. *28*, 382–396.

Peter, H. H., and Feldman, J. D. (1972). Cell-mediated cytotoxicity during rejection and enhancement of allogeneic skin grafts in rats. J. Exp. Med. *135*, 1301–1315.

Rada, C., Lorenzi, R., Powis, S. J., van den Bogaerde, J., Parham, P., and Howard, J. C. (1990). Concerted evolution of class I genes in the major histocompatibility complex of murine rodents. Proc. Natl. Acad. Sci. USA *87*, 2167–2171.

Rebai, N., and Malissen, B. (1983). Structural and genetic analyses of HLA class I molecules using monoclonal xenoantibodies. Tissue Antigens *22*, 107–117.

Schlosstein, L., Terasaki, P. I., Bluestone, R., and Pearson, C. M. (1973). High association of an HL-A antigen, W27, with ankylosing spondylitis. N. Engl. J. Med. *288*, 704–706.

Taurog, J. D., and El-Zaatari, F. A. K. (1988). In vitro mutagenesis of HLA-B27. Substitution of an unpaired cysteine residue in the alpha 1 domain causes loss of antibody-defined epitopes. J. Clin. Invest. *82*, 987–992.

Taurog, J. D., and Lipsky, P. E. (1990). Ankylosing spondylitis and reactive arthritis. In Harrison's Principles of Internal Medicine, J. D. Wilson, E. Braunwald, A. S. Fauci, K. J. Isselbacher, J. B. Martin, R. G. Petersdorf, and R. K. Root, eds. (New York: McGraw-Hill), pp. 1451–1455.

Taurog, J. D., and Moore, P. M. (1986). An unusual motor neuropathy occurring in a patient with quiescent Reiter's disease. Clin. Exp. Rheum. *4*, 147–149.

Taurog, J. D., Lowen, L., Forman, J., and Hammer, R. E. (1988a). HLA-B27 in inbred and non-inbred transgenic mice. Cell surface expression and recognition as an alloantigen in the absence of human β_2-microglobulin. J. Immunol. *141*, 4020–4023.

Taurog, J. D., Argentieri, D. C., and McReynolds, R. A. (1988b). Adjuvant arthritis. Meth. Enzymol. *162*, 339–355.

Taurog, J. D., Hammer, R. E., Maika, S. D., Sams, K. L., El-Zaatari, F. A. K., Stimpson, S. A., and Schwab, J. H. (1990). HLA-B27 transgenic mice as potential models of human disease. In Transgenic Mice and Mutants in MHC Research, I. K. Egorov and C. S. David, eds. (Berlin: Springer-Verlag), pp. 268–275.

Tiwari, J. L., and Terasaki, P. I. (1985). HLA and Disease Associations (New York: Springer-Verlag).

Toivanen, A., and Toivanen, P., eds. (1988). Reactive Arthritis (Boca Raton, Florida: CRC Press).

Weiss, E. H., Kuon, W., Dorner, C., Lang, M., and Riethmuller, G. (1985). Organization, sequence and expression of the HLA-B27 gene: a molecular approach to analyze HLA and disease associations. Immunobiology *170*, 367–380.

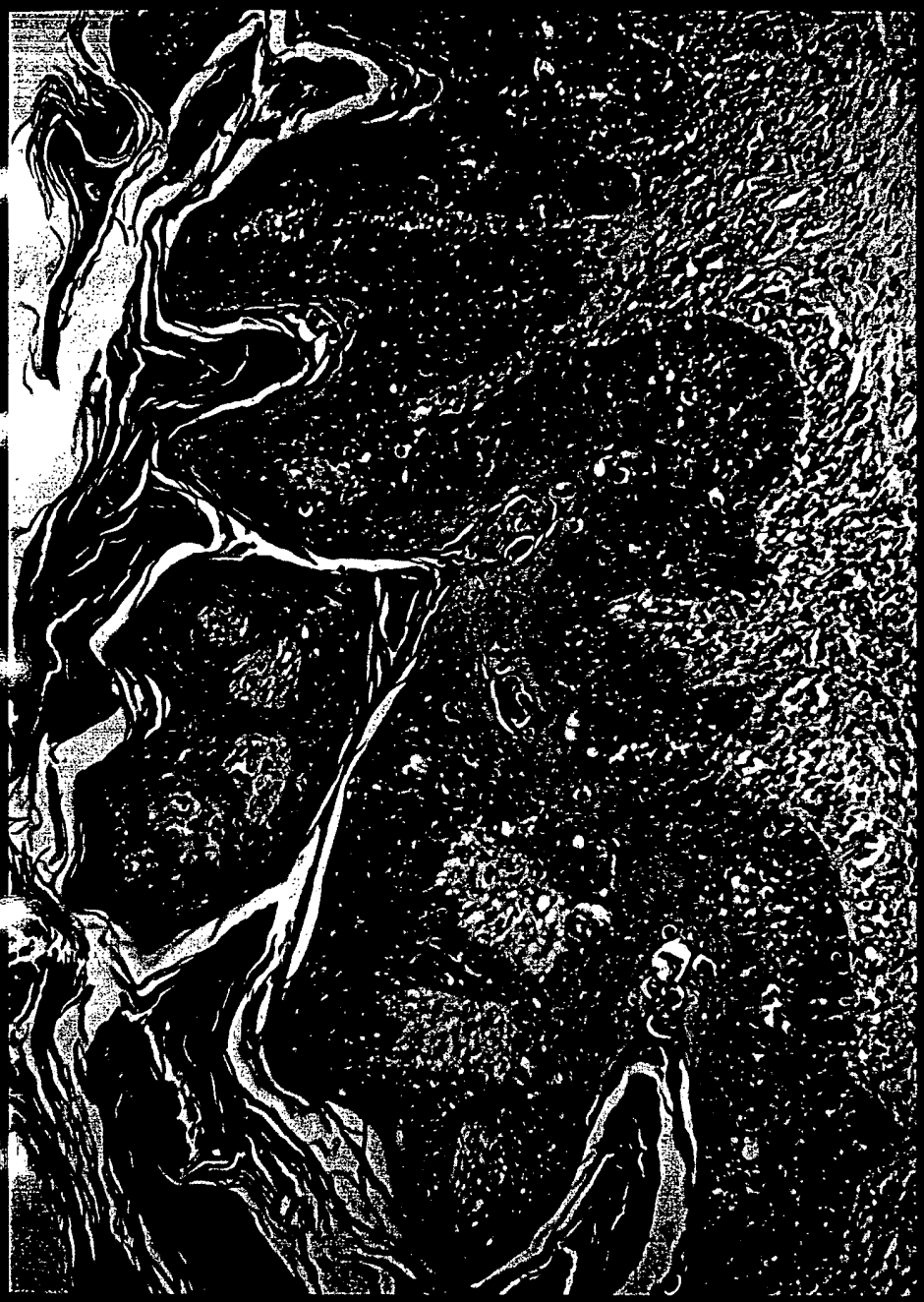
Weiss, E. H., Schliesser, G., Kuon, W., Lang, M., Rietmueller, G., Kievits, F., Ivanyi, P., and Brem, G. (1990). Copy number and presence of human β_2-microglobulin control cell surface expression of HLA-B27 antigen in transgenic mice with a 25 kb B27 gene fragment. In Transgenic Mice and Mutants in MHC Research, I. K. Egorov and C. S. David, eds. (Berlin: Springer-Verlag), pp. 205–213.

Yli-Kerttula, U.-I. (1984). Clinical characteristics in male and female uro-arthritis or Reiter's syndrome. Clin. Rheumatol. *3*, 351–360.

Yu, D. T., Choo, S. Y., and Schaack, T. (1989). Molecular mimicry in HLA-B27-related arthritis. Ann. Intern. Med. *111*, 581–591.

Cell

Volume 63 Number 5 November 30, 1990



Human HLA-B27 Causes Spondyloarthropathies in Transgenic Rats

EXHIBIT C

Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27

MIN AE LEE, MANFRED BÖHM, MARTIN PAUL,
MICHAEL BADER, URSULA GANTEN, AND DETLEV GANTEN
Max Delbrück Center for Molecular Medicine, 13122 Berlin-Buch; and Institute of Clinical Pharmacology, Universitätsklinikum Benjamin Franklin, Free University Berlin, 12200 Berlin, Germany

Lee, Min Ae, Manfred Böhm, Martin Paul, Michael Bader, Ursula Ganten, and Detlev Ganten. Physiological characterization of the hypertensive transgenic rat TGR(mREN2)27. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E919–E929, 1996.—Transgenic techniques represent powerful tools for the study of gene-related mechanisms of diseases such as hypertension, which results from a complex interaction between genetic and environmental factors. The renin-angiotensin system, a biochemical cascade in which renin functions as the key enzyme in the formation of the effector peptide angiotensin II, plays a major role in the regulation of blood pressure. The renin gene, therefore, represents an important candidate gene for hypertension. Because rats are more suited than mice for a number of experimental settings often employed in cardiovascular research, we modified the transgenic technique to generate the transgenic rat strain TGR(mREN2)27 harboring the murine *Ren*-2 gene. These transgenic rats develop fulminant hypertension at an early age despite low levels of renin in plasma and kidney. In addition, high expression of the transgene in a number of extrarenal tissues is associated with increased local formation of angiotensin II. Thus the TGR(mREN2)27 rat represents a model of hypertension with a defined genetic background. Studies on the transgenic rat may not only provide new insights into pathophysiological mechanisms of hypertension in this animal model but also offer the unique possibility to investigate the function and regulation of renin-angiotensin systems in extrarenal tissues. The aim of this review is to compile the knowledge that has been accumulated to date on this transgenic rat and to discuss possible mechanisms responsible for its hypertensive phenotype.

renin-angiotensin system; hypertension

MOST KNOWLEDGE concerning the pathophysiology of primary hypertension has largely been obtained from studies performed in animal models such as the spontaneously hypertensive rat (SHR) and its stroke-prone substrain (SHRSP), which were established by selective breeding of the hypertensive phenotype. As in most cases of primary hypertension in humans, high blood pressure in these animal models is a quantitative trait under polygenic control (61). Apart from rare monogenetic forms of hypertension (43, 70), the genes involved in the hypertensive process remain largely unknown. One approach to identify chromosomal regions containing such genes is to perform linkage studies in crosses between hypertensive rat strains and their normotensive control strains (44, 61). Another approach is to analyze mechanisms by which selected genes influence the development of pathophysiological phenotypes with the use of transgenic techniques (20). The major advantage of transgenic animal models over disease models of unknown etiology is that ensuing pleiotropic phenotypic effects can be attributed to a single genetic change. This is especially important in the study of complex diseases such as hypertension, in which causal effects are difficult to differentiate from secondary effects.

The renin-angiotensin system (RAS) is pivotally involved in the regulation of blood pressure and fluid homeostasis. Therefore, the genes encoding for its components represent important candidate genes for hypertension. In addition to its well-defined role as an endocrine system, there is increasing evidence for locally acting RAS in several tissues, including brain (60), adrenal glands (90), kidney (42), heart (45), and reproductive organs (15). Because renin represents the key enzyme in the cascade leading to the generation of angiotensin II

(ANG II), the murine *Ren*-2 gene was chosen as a candidate gene to generate transgenic rats (51).

TRANSGENIC METHODOLOGY

Significance of Transgenic Techniques

Since its first application in mammals more than a decade ago, the generation of transgenic animals by introducing foreign genes into a host genome has become a very powerful method in experimental biology and medicine. The transgenic approach, based on gain-of-function alterations, has yielded numerous insights into the physiological role of genes under in vivo conditions. In particular, it has provided unique tools to study the tissue-specific or developmental regulation of genes and to analyze gene-related mechanisms of disease (19, 24). The most commonly used species for the generation of transgenic animals are mice because of the availability of several inbred strains with a well-defined genetic background. Although studies on transgenic mice have provided further insight into the role of certain genes involved in cardiovascular homeostasis, such as vasopressin, atrial natriuretic peptide, and the low-density lipoprotein receptor (19), their small size limits their usefulness for a number of experimental settings often employed in hypertension research. Therefore, we decided to apply the transgenic methodology to the rat. The murine *Ren*-2 gene was chosen for the following reasons. *1*) The renin gene represents a major candidate gene for hypertension on the basis of its physiological and pathophysiological role in the regulation of blood pressure. In addition, allelic variants of the renin gene have been shown to cosegregate with the hypertensive phenotype in certain rat strains (37, 62). *2*) Mouse renin is capable of cleaving rat angiotensinogen to produce angiotensin I (ANG I) and increases blood pressure if injected into rats (55, 56). *3*) The murine *Ren*-2 gene differs from other mammalian renin genes in that its protein is unglycosylated and shows a distinct pattern of extrarenal expression (71). Moreover, introduction of the *Ren*-2 gene into the genome of mice has already been demonstrated to result in correct tissue-specific expression (52). Thus, it was hypothesized that overexpression of murine renin in rats would affect blood pressure and allow investigation of those tissues in which local RAS may play an important functional role.

Generation of the Transgenic Rat Line TGR(mREN2)27

A modified version of the protocol etablished for the generation of transgenic mice by pronucleic injection was applied to the rat (28). Female rats derived from a cross between female outbred Sprague-Dawley (SD) rats (Zentralinstitut für Versuchstierkunde, Hannover) and male Wistar-Kyoto (WKY) rats were subjected to hormonal treatment to induce superovulation. At 9–10 wk of age, rats had osmotic minipumps, loaded with 200 μg of a pregnant mare's serum gonadotropin, implanted subcutaneously. Two days later, the rats were injected intraperitoneally with 30–40 units of human chorionic gonadotropin and mated with male SD rats. The following day, the animals were killed, and fertilized oocytes were harvested for microinjection. After the development of pronuclei, ~1 pl of injection buffer containing foreign DNA at a concentration of 1 ng/μl was injected into the larger male pronucleus by use of microinjection apparatus. The injected DNA contained the entire *Ren*-2 gene of DBA/2 mice, including 5.3 kb of the 5′-flanking and 9.5 kb of the 3′-flanking region (51, 52). Manipulated oocytes were transferred to the oviducts of pseudopregnant SD females by injecting medium containing up to 15 oocytes into the infundibulum via retroperitoneal access. Pseudopregnancy was achieved by mating with vasectomized males. Screening of offspring for successful integration of the transgene was performed by Southern blot analysis of *Pvu* II digested genomic DNA obtained from tail biopsies with use of a radioactively labeled *Bam*H I fragment of the *Ren*-2 cDNA. A transgene-positive offspring was identified by the presence of 8.5-kb and 0.8-kb *Ren*-2-specific restriction fragments (51).

Implantation of 37 eggs resulted in eight progeny, of which five carried the transgene (51). Four of the founder animals were successfully bred, and three of them, TGR (mREN2)25, -26, and -27, transmitted the transgene to their progeny. All transgenic founder animals exhibited blood pressure values in the range of 230–265 mmHg compared with 120 mmHg in transgene-negative littermates. The female founder animal 26 was not hypertensive at onset, and further breeding revealed this animal to be mosaic for the transgene. It was not possible to breed these animals to homozygosity because all male offspring were infertile. In contrast to homologous recombination techniques, insertion of foreign DNA by pronucleic injection does not target a specific chromosomal site and occurs at random. The fact that all transgene-positive founder animals were hypertensive and that the hypertensive phenotype cosegregated with the transgene indicates that hypertension development is independent of the transgene insertion site and not caused by a mutation associated with the integration event. The infertility of male TGR(mREN2)26 may be due either to an insertional mutation on the X chromosome or to an autosomal dominant mutation, because the phenotype is already present in the heterozygous state. The transgenic rat strain TGR(mREN2)27 was established by breeding transgene-positive progeny with SD rats. The founder animals were derived from a cross between a female SD × WKY hybrid and a male SD rat and thus derived one-fourth of their genetic material from the WKY genome. Because further breeding was performed by using SD rats and selecting for the transgene, it can be calculated that the transgene was completely bred into the genetic background of SD rats after 8–10 generations (61). Therefore the SD rat derived from the Hannover colony repesents the appropriate control strain for TGR(mREN2)27 rats.

CHARACTERIZATION OF PHENOTYPE

Development of Blood Pressure, Body Weight, and Survival Rates

The presence of an additional renin gene in TGR(mREN2)27 rats is associated with the development of severe hypertension (51). In heterozygous animals, hypertension was evident at 4–5 wk of age, reaching maximum values of up to 240 mmHg in males and up to 200 mmHg in females at 8–9 wk. Blood pressure values in SD rats ranged between 115 and 140 mmHg (Fig. 1). The phase of established hypertension was followed by a decrease in blood pressure by 20–30 mmHg in male, and by 40–60 mmHg in female TGR(mREN2)27 rats between 20 and 24 wk of age. Doubling of gene dose in homozygous rats led to even higher blood pressure values, reaching 290 mmHg in males and 250 mmHg in females (Fig. 1). In heterozygous transgenic animals, increase in body weight, an indicator of thriving, was comparable to that of SD rats. Homozygous animals, however, displayed a lack of weight gain, which was paralleled by an increased mortality rate (Fig. 2). Morphological changes consistent with hemorrhagic stroke were occasionally found on autopsy predominantly in homozygous TGR(mREN2)27 rats.

Plasma RAS

On the basis of most studies conducted so far on the plasma RAS in the transgenic rat, one of the most interesting observations after the introduction of an additional renin gene in the rat is that renin in plasma and kidney is reduced, whereas prorenin is elevated (51). The suppression of renin and the prorenin elevation were both present at 5 wk of age before hypertension was fully established (Fig. 3, *A-D*). Converting-enzyme activity in plasma was similar between heterozygous TGR(mREN2)27 and SD rats (Fig. 3, *E* and *F*), although homozygosity for the transgene has been shown to be associated with a reduction in plasma-converting enzyme (10, 39). Plasma levels of ANG I were decreased to 20% of control values in transgenic females but were only slightly lower in transgenic than in control males (Fig. 4, *A* and *B*). Similarly, ANG II was reduced in transgenic females compared with female controls but was not different between transgenic and control males (Fig. 4, *C* and *D*). Altogether, ANG I and ANG II were significantly higher in male than in female transgenic rats. This sexual dimorphism was not present in control animals. Plasma angiotensinogen was slightly decreased in both female and male transgenic rats compared with controls (Fig. 4, *E* and *F*).

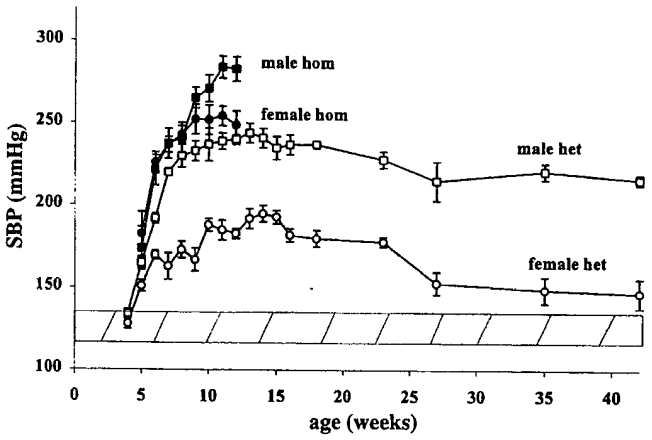


Fig. 1. Development of systolic blood pressure (SBP) measured by tail cuff plethysmography in heterozygous (het) and homozygous (hom) transgenic rat (TGR) (mREN2)27 rats of both sexes. Values are means ± SD; n = 10/group. $P < 0.005$ at 5 wk and $P < 0.0005$ beyond 5 wk of age for all groups vs. normotensive Sprague-Dawley (SD) rats matched for age and sex, respectively, except for female heterozygous rats, $P < 0.0005$ only until 21 wk. Hatched bar, range of blood pressure values in control animals.

Although most investigations on TGR(mREN2)27 rats have confirmed the presence of reduced circulating renin (3, 26, 39, 40, 47, 50, 59, 64, 66), some studies have reported increased plasma renin levels in TGR(mREN2)27 rats (10, 80, 81). This discrepancy clearly requires further exploration, as it concerns the role of circulating renin for the hypertensive phenotype. Several factors must be considered when the levels of circulating renin reported in these studies are interpreted. First, phenotypic effects of the transgene may vary because of differences in the genetic background of the host genome. Whitworth et al. (88) recently demonstrated that crossbreeding of TGR(mREN2)27 rats with Edinburgh SD rats resulted in a change of the phenotype resembling malignant hypertension. Because this phenotype was not observed during crossbreeding of TGR(mREN2)27 rats with Hannover SD rats under identical environmental conditions, the phenotypic shift was attributed to the genetic diversity within outbred SD rats (88). Thus differences in the genetic makeup between different colonies of SD rats may also affect plasma renin levels, emphasizing the need for appropriate controls. Second, plasma renin levels vary depending on gender, age, and zygosity of the transgenic rats. Moreover, high blood pressure in homozygous transgenic rats necessitates antihypertensive treatment during pregnancy. Prenatal treatment with a converting-enzyme inhibitor in SHR has been demonstrated to attenuate development of hypertension in adult rats even after withdrawal of the drug (89). It is possible that similar epigenetic influences in homozygous TGR(mREN2)27 rats may affect the phenotype. Third, prorenin can be activated in vitro after exposure to cold or low pH (54), and artifactual activation during sample processing may account for increased renin, especially in the transgenic rat, where circulating prorenin is high. In addition, species-specific differences in plasma renin levels measured may occur depending on the pH, as well as the source of renin substrate used (23, 81).

Sexual Dimorphism

TGR(mREN2)27 rats exhibit a marked sexual dimorphism with respect to the hemodynamic phenotype because males have higher blood pressure than females. Therefore, the transgenic rat offers the possibil-

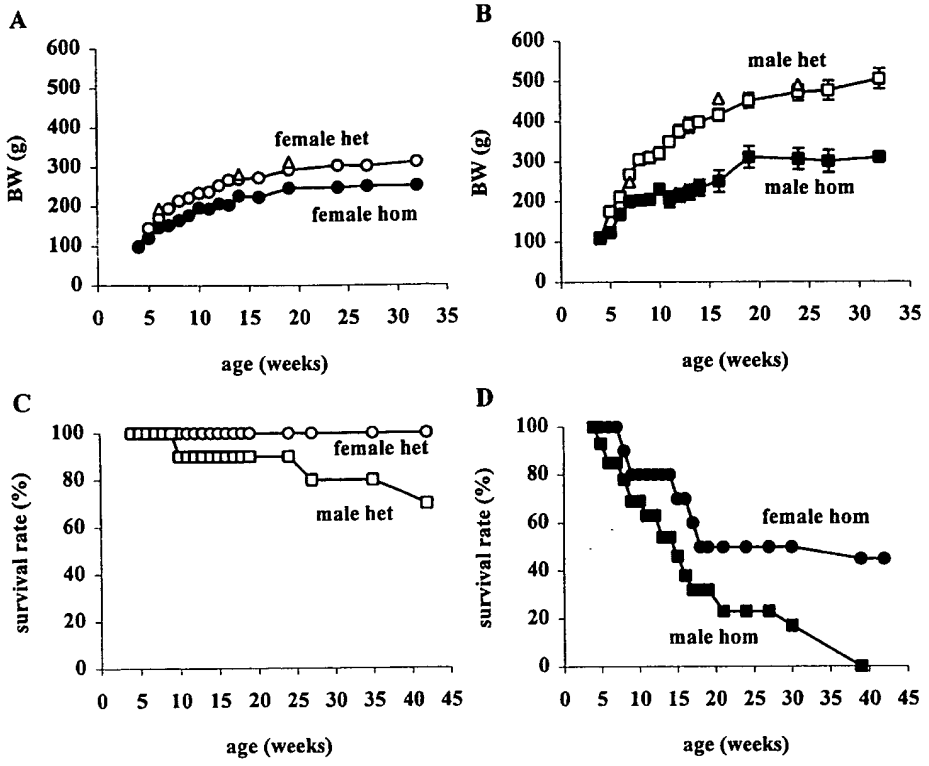


Fig. 2. Development of body weight in female (*A*) and male (*B*) heterozygous (het) and homozygous (hom) transgenic rats. Triangles, values for age-matched SD rats. Values are means ± SD; n = 10/group. $P < 0.005$, or 0.0005 beyond 7 wk of age, for comparison between heterozygous and homozygous rats. *C* and *D*: survival rates in TGR(mREN2)27 rats.

ity to study the role of the renin gene in sex-specific differences of hypertension, a phenomenon that is also observed in other rat strains (21) and in humans (13). Interestingly, gender-dependent phenotypic differences were also observed for certain components of the plasma RAS. Higher plasma angiotensinogen, observed in both transgenic and control males, may be attributed to androgens, which have been shown to stimulate hepatic angiotensinogen gene transcription (11). However, prorenin, ANG I, and ANG II were higher only in transgenic males compared with females, pointing to a transgene-related mechanism for this sex difference. *Ren*-2 gene expression is positively modulated by androgens (82). Furthermore, treatment of female DBA/2 mice with testosterone leads to an enhanced transcriptional activity of the *Ren*-2 gene in the submandibular gland (87), whereas treatment of young female TGR(mREN2)27 rats with testosterone has been shown to increase blood pressure to the levels of males (2). These findings indicate that androgens influence blood pressure by affecting *Ren*-2 gene expression. Peters et al. (59) demonstrated that most of the prorenin, as well as a substantial part of renin, in the circulation of TGR(mREN2)27 rats is of transgene origin. Therefore, androgen-stimulated *Ren*-2 gene expression may account for higher prorenin levels in male transgenic rats. Relatively higher levels of ANG I and ANG II in male than in female TGR(mREN2)27 rats may result from enhanced kinetics between rat angiotensinogen and mouse renin (81).

Tissue-Specific Phenotypic Characteristics

The transgene is expressed in a large number of tissues, with the highest expression found in adrenal gland, thymus, brain, and gastrointestinal and urogenital tracts, whereas *Ren*-2 gene expression in the kidney is low (51, 92). Comparison of the *Ren*-2 gene expression pattern between DBA/2 mice and transgenic rats demonstrates maintenance of correct tissue specificity except for the submandibular gland, where no *Ren*-2 transcripts were detectable in the transgenic rat despite high expression in the mouse (51, 92). This is most likely due to the presence of trans-acting factors in the murine submandibular gland, because the transgene includes 5′-flanking sequence elements that have been shown to confer tissue-specific expression of a reporter gene in mice lacking an endogenous *Ren*-2 gene in their genome (72). Because *Ren*-2 gene expression occurs in tissues in which low renin expression is observed in normal rats, overexpression of the transgene, rather than ectopic expression, may lead to activation or interference with preexistent local RAS. This renders TGR(mREN2)27 rats a suitable model for studying the regulation of renin gene expression and its role in cardiovascular disease at these sites.

Adrenal gland. The adrenal gland is the major site of *Ren*-2 gene expression in the transgenic rat (3, 51, 91). Overexpression of the transgene is accompanied by an increased mineralocorticoid excretion in young animals during the phase of hypertension development (66). Because the circulating RAS, a major stimulator of

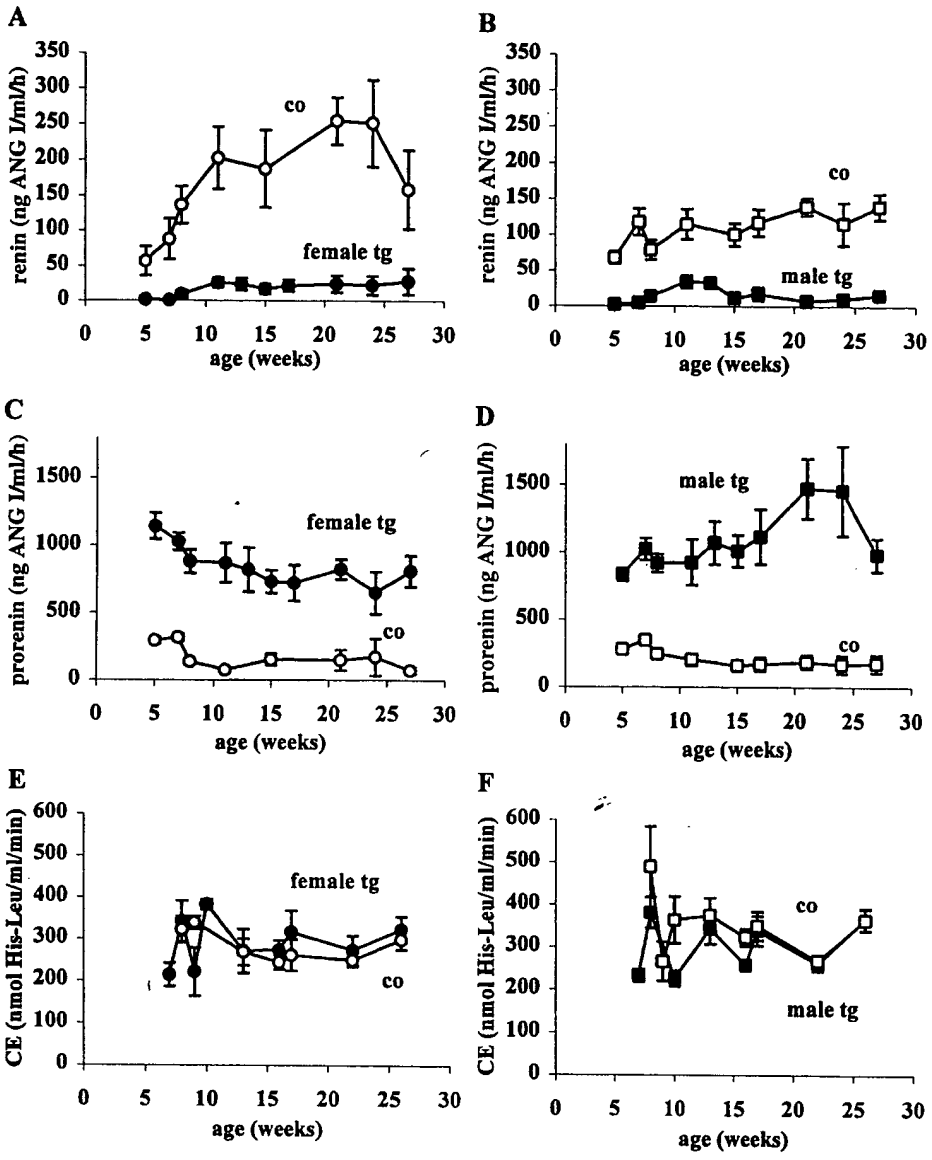


Fig. 3. Plasma renin and prorenin in female (*A*, *C*) and male (*B*, *D*) heterozygous transgenic (tg) and control rats (co) from 5 to 27 wk of age determined by indirect radioimmunoassay at pH 7.2 and after trypsin activation, respectively. Plasma converting enzyme activity (CE) in female (*E*) and male (*F*) heterozygous TGR(mREN2)27 rats. Values are means ± SD; $n = 10$/group. $P < 0.005$ or 0.0005 at all time points for comparison of renin and prorenin between transgenic and control animals.

aldosterone release, is suppressed, an activated adrenal RAS might be responsible for the enhanced steroid metabolism. Indeed, in addition to adrenocorticotropic hormone and potassium, ANG II functions as a major regulator of steroid synthesis in the adrenal cortex, and it is increased in the adrenal gland of TGR(mREN2)27 rats (10, 39, 50). Furthermore, renin has been localized in mitochondrial dense bodies where steroidogenesis takes place, suggesting that locally produced ANG II may mediate the stimulation of basal aldosterone synthetase expression in the adrenal cortex of TGR(mREN2)27 rats (63, 67). Sodium restriction in transgenic rats was shown to selectively stimulate adrenal renin and to increase aldosterone synthesis, whereas the rise in plasma and renal renin was blunted compared with SD rats (64). This stimulation was abolished by AT_1 receptor blockade, indicating that mineralocorticoid synthesis in the transgenic rat is mediated by the AT_1 recptor (85). Renin and prorenin are secreted by isolated glomerulosa cells and are mainly of transgene origin, as demonstrated by immunoprecipitation with a mouse renin-specific antibody (59). The adrenal gland also appears to be the major source for circulating prorenin, because adrenalectomy caused a sustained decrease in plasma prorenin (3, 80). Moreover, ANG II has been demonstrated to stimulate secretion of renin, but not prorenin, in vitro. This implies different regulatory mechanisms for renin and prorenin secretion and indicates that adrenal renin is not subject to a negative feedback control by ANG II (59). The observation that the adrenal RAS is activated suggests that increased sodium and water retention due to oversecretion of mineralocorticoids may be responsible for hypertension in TGR(mREN2)27 rats. However, treatment with an aldosterone antagonist does not reduce or prevent hypertension, providing evidence that mineralocorticoids are not the major pathogenetic factor in the development of hypertension in TGR(mREN2)27 rats

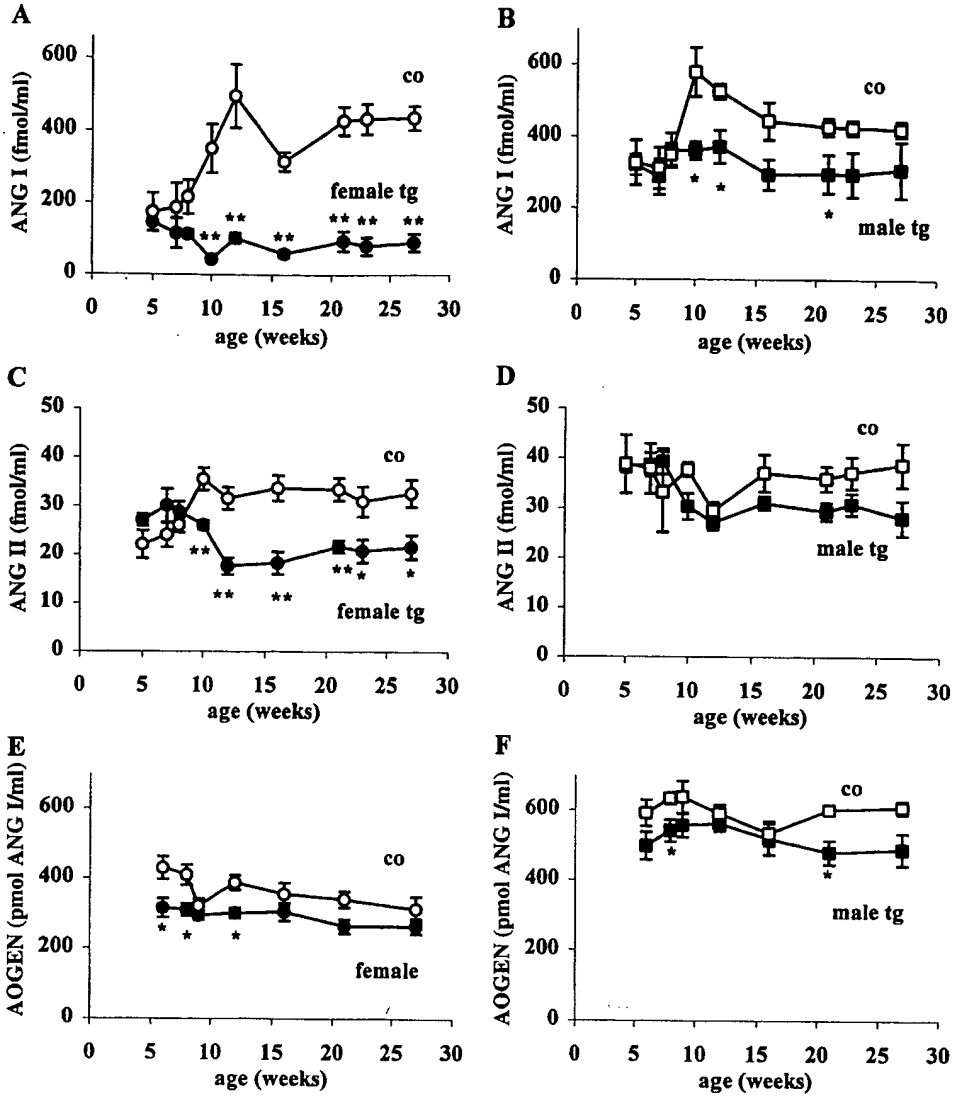


Fig. 4. Plasma angiotensin I (ANG I), angiotensin II (ANG II), and angiotensinogen (AOGEN) in female (*A*, *C*, and *E*) and male (*B*, *D*, and *F*) heterozygous transgenic (tg) and control (co) rats. Values are means ± SD; n = 10/group. $^{*}P < 0.05$ and $^{**}P < 0.005$ vs. control.

(66). This raises the possibility that other tissues that overexpress renin, such as heart, vasculature, or brain, may contribute to the hypertensive phenotype by yet undefined mechanisms.

Heart and vasculature. Growing evidence supports the existence of a self-contained cardiac angiotensin system that may be involved in adaptive processes associated with cardiac hypertrophy (45). Modulation of the expression of components of the RAS, such as converting-enzyme, angiotensinogen, and ANG II receptors, has been demonstrated in several models of cardiac overload (45, 76). Cardiac renin expression has also been demonstrated, albeit at very low levels (18, 58). Isolated cardiomyocytes have been shown to release ANG II in response to mechanical stretch (65). Furthermore, ANG II has been demonstrated to exert growth-promoting effects on isolated chicken myocytes (5) and to induce expression of protooncogenes in smooth muscle cells in vitro (53). Expression of protooncogenes is thought to be one mechanism by which reprogramming of cardiac gene expression in response to chronic volume overload is achieved (31, 68). Recruitment of the cardiac RAS may thus play an important role in pathogenesis of cardiac hypertrophy.

In the transgenic rats, the *Ren*-2 gene is also expressed in the heart and the vasculature, and this expression is accompanied by increased tissue concentrations of ANG II (10, 39, 92). Furthermore, Hilgers et al. (26) demonstrated an enhanced synthesis and release of ANG I and ANG II in isolated perfused hindlimbs of nephrectomized transgenic rats, an experimental setting where blood-borne renin cannot contribute to angiotensin biosynthesis. Transgenic rats develop pathomorphological changes of the cardiovascular system such as myocardial hypertrophy, which is accompanied by an increase in connective tissue and perivascular fibrosis (3). Vascular alterations include increased media thickness and hypertrophied smooth muscle cells of the aorta, as well as an increased media-to-lumen ratio without vascular growth of the mesenteric vasculature, which is consistent with remodeling (3, 79). In addition, functional and biochemical changes similar to those observed in human heart failure have been described in TGR(mREN2)27 rats, including down-

regulation of β-adrenergic receptors accompanied by a reduced positive inotropic response to isoproterenol (9, 78), as well as an increased expression of the gap-function protein, Connexin 40, the stimulation of which is thought to be a compensatory mechanism to increase conduction velocity (7). Moreover, endothelium-dependent contraction in response to the L-arginine analogue nitro-L-arginine methyl ester (L-NAME) in coronary arteries of transgenic rats is decreased, suggesting a reduced basal endothelial-derived nitric oxide release (83).

The question as to whether these changes are solely due to the elevated blood pressure, or whether local activation of the RAS is an independent factor contributing to the cardiovascular pathology in this model, remains uncertain. Enhanced local formation of ANG II, however, is consistent with an activated cardiac and vascular RAS, supporting the notion of its pathogenetic relevance in the transgenic rat. Cardiac dysfunction in TGR(mREN2)27 rats is more pronounced than in SHR, which exhibit a similar hemodynamic phenotype with regard to onset and degree of hypertension (57). Moreover, specific inhibitors of the RAS, such as converting-enzyme inhibitors or ANG II receptor antagonists, are capable of inducing regression of heart hypertrophy, even at low doses that only cause a slight reduction in blood pressure, whereas direct vasodilators fail to exhibit similar cardioprotective effects despite efficient blood pressure reduction (4, 8, 39).

Kidney. Despite the presence of an additional renin gene in the genome of TGR(mREN2)27 rats, renin activity in the kidney is markedly reduced (51). Whereas suppression of the endogenous rat renin gene is similar in hetero- and homozygous transgenic rats, *Ren*-2 gene expression is higher in homozygous animals and, therefore, transgene dose dependent (39). Interestingly, renal ANG II content is reduced in heterozygous animals compared with controls, whereas in homozygous animals it is unchanged or even increased, correlating with renal *Ren*-2 gene expression (10, 39). Thus renal ANG II, which appears to be inappropiately high at least in homozygous transgenic animals, may result from enhanced kinetics between mouse renin and rat renal angiotensinogen (81). Because intrarenal ANG II has been shown to exert a negative regulatory influence on renin expression (32), locally generated ANG II in the transgenic rat may participate in the suppression of the endogenous renin gene independently of blood pressure.

The role of the kidney in the development and maintenance of hypertension is well established, and several lines of evidence indicate that an intrarenal RAS is an important regulator of renal hemodynamics and function (42). TGR(mREN2)27 rats exhibit functional and structural changes of the kidney that are commonly observed in hypertension, such as a shift of the pressure-natriuresis relationship toward higher arterial pressure, reduced glomerular filtration rate, and albuminuria. The albuminuria appears as early as 8 wk of age and is associated with glomerulosclerosis (3, 22, 75). ANG II is a powerful modulator of sodium reabsorption and has recently been shown to upregulate proximal tubule expression of the AT_1 receptor (12). Therefore, elevated tubular sodium reabsorption that accompanies the resetting of the pressure-natriuresis response toward higher levels of renal perfusion pressure may be attributed to locally generated ANG II in the kidney in TGR(mREN2)27 rats (22). In addition, a study on the tubuloglomerular feedback mechanism suggests an increased responsiveness in the transgenic rat, which was attenuated to a greater extent after AT_1 receptor blockade than after mechanical reduction of renal perfusion pressure (46). This observation indicates that the increased tubuloglomerular feedback responsiveness is ANG II dependent.

ANG II receptor blockade, but not the combined regimen of reserpine, hydrochlorothiazide, and hydralazine, has been shown to limit glomerular injury in rats with reduced renal mass, implying that remnant glomerular function and structure depend on reduction of ANG II activity (38). Similarly, antihypertensive treatment with inhibitors of the RAS, but not hydralazine, is capable of halting the progression of glomerulosclerosis in TGR(mREN2)27 rats (8, 39), suggesting that these structural alterations may not solely be hypertension related, but primary events caused by interference or activation of the renal RAS because of the presence of the transgene.

Central and peripheral nervous system. Expression of the transgene in the brain of TGR(mREN2)27 rats is already present in newborn animals and appears to be developmentally regulated. In the hypothalamus, *Ren*-2 gene expression persists in adult animals, whereas it is downregulated in the medulla oblongata (92). This is accompanied by a marked elevation of ANG II in the brain of transgenic rats, indicating the presence of an activated RAS and suggesting a role for the central nervous system in the pathogenesis of hypertension in this strain (10, 69). In normal rats, angiotensinogen, the only known substrate for renin, is highly expressed in glial cells (60). Likewise, converting-enzyme and ANG II receptors have been readily demonstrated, whereas renin, the cellular localization of which is unknown, is minutely expressed within the central nervous system (1, 17).

ANG II is an important modulator of the sympathetic nervous system and is involved in the regulation of thirst and the release of vasopressin (60). Although an enhanced interaction between vascular ANG II and the sympathetic nervous system appears to be absent in TGR(mREN2)27 rats (27), central modulation of sympathetic tone may be one of the mechanisms by which the RAS in the brain participates in the hypertensive process. Interestingly, it has been demonstrated that the hypertensive effect, as well as the central vasopressin release in response to centrally administered ANG II in transgenic rats, is absent (48). Furthermore, the central distribution of vasopressin has been shown to be altered in TGR(mREN2)27 rats, suggesting an interaction between the central RAS and the vasopressin axis (48). Moriguchi et al. (48) demonstrated that intracerebrovascular administration of an ANG II-

specific antibody led to a decrease, whereas application of an ANG-(1—7) antibody led to an increase, in blood pressure in TGR(mREN2)27 but not in SD rats, suggesting opposing actions of ANG II and ANG-(1—7) in the brain of transgenic rats and supporting the contention of central ANG II in the pathogenesis of hypertension in the transgenic rat (49). Whether altered circadian rhythms of blood pressure may be related to overexpression of the transgene in the brain awaits further investigation (41).

MECHANISMS OF HYPERTENSION IN TGR(mREN2)27

TGR(mREN2)27 rats develop fulminant hypertension from the presence of an additional renin gene in their genome. Several lines of evidence indicate that the hypertensive phenotype is indeed determined by the transgene and not due to an insertional mutation. First, all transgenic founder animals were hypertensive irrespective of the transgene insertion site within the genome. Second, hypertension cosegregates with the transgene, and, third, hypertensive transgenic rats respond with great sensitivity to antihypertensive treatment with converting-enyzme inhibitors or the ANG II receptor antagonist, indicating that hypertension is ANG II dependent (4, 8, 39, 51).

One of the most striking features of TGR(mREN2)27 is the suppression of the circulating renin, which appears to exclude the plasma RAS as the cause for the hypertensive phenotype (51). High expression of the transgene is associated not only with an increased renin activity but also with an enhanced local formation of ANG II, indicating that the transgene is translated into a functional protein (10, 26, 39, 51). Furthermore, tissue-specific differential modulation of gene expression of RAS components suggests the presence of different regulatory and functional properties of the RAS at these sites (39). Local formation of ANG II in organs involved in cardiovascular homeostasis supports the idea of a causal role of extrarenal RAS in the pathogenesis of hypertension and structural alterations, such as cardiovascular hypertrophy and glomerulosclerosis.

The transgenic rat, therefore, demonstrates in a very intriguing way that the RAS can be causally involved in the hypertensive process, even in absence of elevated circulating renin. This represents probably one of the most convincing pieces of evidence for a physiological significance of tissue-resident RAS and suggests that they may be involved in the pathogenesis of other forms of hypertension associated with normal or low plasma renin. This contention is in agreement with the commonly observed lack of correlation between the antihypertensive effect of converting-enzyme inhibitors and plasma renin or converting-enzyme levels before and after treatment (84, 86). The findings in the transgenic rat confirm the hypothesis that plasma levels of the components of the RAS may not always be an appropriate indicator for the activity of the system, because they do not allow any conclusions as to the activity of local RAS.

Another characteristic feature of the transgenic rat is the elevated prorenin that is mainly of mouse origin and, therefore, transgene derived (59). The physiological significance of circulating prorenin is still uncertain, and the question whether it is intrinsically active, or undergoes local activation in vivo, is controversially discussed (54). Exogenously applied prorenin is not activated in the circulation of SHR and monkeys but appears to be sequestrated and activated by the kidney without affecting blood pressure (29, 34). Similarly, it has been suggested that prorenin can be sequestrated from the coronary circulation in humans (73). The finding of prorenin-converting enzymes in rat aorta and kidney, as well as in human neutrophil cells (16, 77), has led to the hypothesis that circulating prorenin may be taken up locally and activated by lysosomal proteases or an acid pH milieu. Subsequent intracellular generation of ANG II may then influence local perfusion or exert trophic effects on the vascular wall. Interestingly, a recently established new transgenic rat strain, which harbors the *Ren*-2 gene fused to a liver-specific promoter, exhibits high plasma prorenin because of hepatic secretion and develops hypertension as well as cardiac hypertrophy (6). Because active *Ren*-2 gene-derived renin was detectable, this model suggests that extrarenal prorenin can be activated in vivo, leading to hypertension (6). However, whether high circulating prorenin in TGR(mREN2)27 rats merely represents an epiphenomenon or contributes to the cardiovascular phenotype requires further detailed investigation.

CONCLUSION AND OUTLOOK

In summary, TGR(mREN2)27 represents the first rat model of hypertension in which the genetic defect is precisely defined. Fulminant hypertension in the presence of suppressed plasma RAS and high renin expression in extrarenal tissues suggests that activated extrarenal RAS might be responsible for the hypertensive phenotype. Regulated expression of the transgene in concert with *cis*- and *trans*-regulatory factors appears to have tissue-specific effects on preexisting local RAS. The *Ren*-2 gene gives rise to a functional protein that leads to increased local formation of ANG II, which then acts on target tissues in a paracrine or autocrine manner. This local ANG II formation may be a key factor for the changes in cardiovascular morphology that appear to be partially independent from blood pressure.

The importance of ANG II in the regulation of blood pressure, fluid, and electrolyte balance, as well as cardiovascular growth, is well recognized. TGR(mREN2)27 rats overexpress renin in a number of extrarenal tissues that are not obviously linked with cardiovascular function, such as thymus, and gastrointestinal and urogenital tracts. This provides the unique opportunity to investigate the functional role of ANG II in these tissues. Among the more recent evidence for multiple and yet undefined functions of ANG II is the intricate developmental and tissue-specific regulation of the expression of ANG II receptors, notably the AT_2 recep-

tor, which is highly expressed in embryo and brain (33), as well as the existence of at least two converting-enzyme isoforms in testis and intestine, respectively, in addition to the endothelial isoform (14, 36). To explore such functions, the transgenic gain-of-function model should be complemented with loss-of-function models using homologous recombination, as recently demonstrated for several RAS components (74). Targeted disruption of the converting-enzyme gene in mice is associated with a slight reduction in blood pressure and male infertility, providing direct evidence for a role of angiotensin-converting enzyme in male gonadal function (35). Two reports on the homozygous deletion of the AT_2 receptor gene in mice describe changes in exploratory behavior as well as neurophysiological parameters, such as body temperature and algesia (25, 30). These findings further illustrate the wide spectrum of the functional properties of ANG II. In addition, mutant mice lacking the AT_2 receptor show an increased pressure response to ANG II, suggesting mutually counteracting hemodynamic effects between the AT_1 and the AT_2 receptors (25, 30).

Although TGR(mREN2)27 rats provide a large amount of information on the role of the RAS in cardiovascular disease, they cannot be considered as a genuine model of human hypertension, which is polygenic in nature. They do provide, however, a sophisticated approach to analyze phenotypic consequences of defined genetic alteration in an in vivo system. Despite the fact that the exact mechanisms underlying the phenotype are still uncertain, the transgenic rat paradigmatically demonstrates that alterations within extrarenal RAS may result in a phenotype characterized by hypertension and pathological changes within the cardiovascular system. Therefore one may speculate that similar alterations in extrarenal renin under yet undefined physiopathological circumstances may also be of relevance for some forms of human hypertension. Enhancing our knowledge regarding the transgenic rat may ultimately lead to a better understanding of the mechanisms by which the RAS contributes to the pathogenesis of hypertension, providing the basis for future studies in humans.

We thank Claire Bayer for expert technical assistance; Dr. Jürgen Bachmann for blood pressure measurements; and Dr. Klaus Lindpaintner, Dr. Speranza Rubattu, and Dr. Chris E. Talsness for helpful discussions.

This work was supported by Deutsche Forschungsgemeinschaft; National Heart, Lung, and Blood Institute; the European Community (Trans-Gen-Eur); and Bristol-Meyers-Squibb (Princeton, NJ).

Address for reprint requests: M. Paul, Institute of Clinical Pharmacology, Universitätsklinikum Benjamin Franklin, Free University Berlin, Hindenburgdamm 30, 12200 Berlin, Germany.

REFERENCES

1. **Allen, A. M., S. Y. Chai, P. M. Sexton, S. J. Lewis, A. J. M. Verberne, B. Jarrott, W. J. Louis, J. Clevers, M. J. McKinley, G. Paxinos, and F. A. O. Mendelsohn.** Angiotensin II receptors and angiotensin converting enzyme in the medulla oblongata. *Hypertension Dallas* 9: 197–205, 1987.
2. **Bachmann, J., U. Ganten, F. Zimmermann, J. J. Mullins, G. Stock, and D. Ganten.** Sexual dimorphism of blood pressure in transgenic rats TGR(mREN2)27 harboring the murine Ren-2 gene. In: *Colloque INSERM, Genetic Hypertension,* edited by J. Sassard. Paris: John Libbey Eurotext, 1992, vol. 218, p. 353–355.
3. **Bachmann, S., J. Peters, E. Engler, D. Ganten, and J. Mullins.** Transgenic rats carrying the mouse renin gene—morphological characterization of a low renin hypertension model. *Kidney Int.* 41: 24–36, 1992.
4. **Bader, M., Y. Zhao, M. Sander, M. Lee, J. Bachmann, M. Böhm, B. Djavidani, J. Peters, J. J. Mullins, and D. Ganten.** Role of tissue renin in the pathophysiology of hypertension in TGR(mREN2)27 rats. *Hypertension Dallas* 19: 681–686, 1992.
5. **Baker, K. M., and J. F. Aceto.** Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells. *Am. J. Physiol.* 259 (*Heart Circ. Physiol.* 28): H610–H618, 1990.
6. **Barrett, G., D. Ogg, J. Peters, S. Fleming, C. Whitworth, and J. J. Mullins.** Hypertension and cardiac hypertrophy as a result of high circulating prorenin levels (Abstract). *J. Hypertens.* 12: S173, 1994.
7. **Bastide, B., L. Neyses, D. Ganten, M. Paul, K. Willecke, and O. Traub.** Gap junction protein connexin-40 is preferentially expressed in vascular endothelium and conductive bundles of rat myocardium and is increased under hypertensive conditions. *Circ. Res.* 73: 1138–1149, 1993.
8. **Böhm, M., M. A. Lee, R. Kreutz, S. Kim, M. Schinke, B. Djavidani, J. Wagner, M. Kaling, W. Wienen, M. Bader, and D. Ganten.** Angiotensin II receptor blockade in TGR(mREN2)27: effects of renin-angiotensin system gene expression and cardiovascular functions. *J. Hypertens.* 13: 891–899, 1995.
9. **Böhm, M., M. Moll, B. Schmid, M. Paul, D. Ganten, M. Castellano, and E. Erdmann.** Beta-adrenergic neuroeffector mechanisms in cardiac hypertrophy of renin transgenic rats. *Hypertension Dallas* 24: 653–662, 1994.
10. **Campbell, D. J., P. Rong, A. Kladis, B. Rees, and S. L. Skinner.** Angiotensin and bradykinin peptides in the TGR(mREN-2)27 rat. *Hypertension Dallas* 25: 1014–1020, 1995.
11. **Chen, Y.-F., A. J. Naftilan, and S. Oparil.** Androgen-dependent angiotensinogen and renin messenger RNA expression in hypertensive rats. *Hypertension Dallas* 19: 456–463, 1992.
12. **Cheng, H. F., B. N. Becker, K. D. Burns, and R. C. Harris.** Angiotensin II upregulates type-1 angiotensin II receptors in renal proximal tubule. *J. Clin. Invest.* 95: 2012–2029, 1995.
13. **Dawber, T. R.** *The Framingham Study. The Epidemiology of Atherosclerotic Disease.* Cambridge, MA: Harvard University Press, 1980.
14. **Deddish, P., J. Wang, B. Michel, P. W. Morris, N. O. Davidson, R. A. Skidgel, and E. G. Erdös.** Naturally occurring active N-domain of human angiotensin I-converting enzyme. *Proc. Natl. Acad. Sci. USA* 91: 7807–7811, 1994.
15. **Do, Y. S., A. Sherrod, R. A. Lobo, R. J. Paulson, T. Shinagawa, S. Chen, S. Kjos, and W. A. Hsueh.** Human ovarian theca cells are a source of renin. *Proc. Natl. Acad. Sci. USA* 85: 1957–1961, 1988.
16. **Dzau, V. J.** Possible prorenin activating mechanisms in the blood vessel wall. *J. Hypertens. Suppl.* 5, *Suppl.* 2: S15–S18, 1987.
17. **Dzau, V. J., J. Ingelfinger, R. E. Pratt, and K. E. Ellison.** Identification of renin and angiotensinogen messenger RNA sequences in mouse and rat brains. *Hypertension Dallas* 8: 544–548, 1986.
18. **Dzau, V. J., and R. N. Re.** Evidence for the existence of renin in the heart. *Circulation* 75, *Suppl.* I: I134-I136, 1987.
19. **Field, L. J.** Cardiovascular research in transgenic animals. *Trends Cardiovasc. Med.* 1: 141–146, 1991.
20. **Ganten, D., K. Lindpaintner, U. Ganten, J. Peters, F. Zimmermann, M. Bader, and J. Mullins.** Transgenic rats: new animal models in hypertension research. *Hypertension Dallas* 17: 843–855, 1991.
21. **Ganten, U., G. Schröder, M. Witt, F. Zimmermann, D. Ganten, and G. Stock.** Sexual dimorphism of blood pressure in spontaneously hypertensive rats: effects of anti-androgen treatment. *J. Hypertens.* 7: 721–726, 1989.
22. **Gross, V., R. J. Roman, and A. W. Cowley.** Abnormal pressure-natriuresis in transgenic renin gene rats. *J. Hypertens.* 12: 1029–1034, 1994.

23. **Gutkowska, J., P. Corvol, A. F. S. Figueiredo, T. Inagami, J. Bouhnik, and J. Genest.** Kinetic studies of rat renin and tonin on purified rat angiotensinogen. *Can. J. Biochem. Cell Biol.* 62: 137–142, 1984.
24. **Hanahan, D.** Transgenic mice as probes into complex systems. *Science Wash. DC* 246: 1265–1275, 1989.
25. **Hein, L., G. S. Barsh, R. E. Pratt, V. J. Dzau, and B. K. Kobilka.** Behaviourial and cardiovascular effects of disrupting the angiotensin II type-2 receptor gene in mice. *Nature Lond.* 377: 744–747, 1995.
26. **Hilgers, K. F., J. Peters, R. Veelken, M. Sommer, G. Rupprecht, D. Ganten, F. C. Luft, and J. F. E. Mann.** Increased vascular angiotensin formation in female rats harboring the mouse Ren-2 gene. *Hypertension Dallas* 19: 687–691, 1992.
27. **Hilgers, K. F., R. Veelken, I. Kreppner, D. Ganten, F. C. Luft, H. Geiger, and J. F. E. Mann.** Vascular angiotensin and the sympathetic nervous system: do they interact? *Am. J. Physiol.* 267 (*Heart Circ. Physiol.* 36): H187–H194, 1994.
28. **Hogan, B., F. Costantini, and E. Lacy.** *Manipulating the Mouse Embryo.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1986.
29. **Hosoi, M., S. Kim, T. Yamauchi, T. Watanabe, K. Murakami, F. Suzuki, A. Takahashi, Y. Nakamura, and K. Yamamoto.** Similarity between physicochemical properties of recombinant rat prorenin and native inactive renin. *Biochem. J.* 275: 727–731, 1991.
30. **Ichiki, T., P. A. Labosky, C. Shiota, S. Okuyama, Y. Imagawa, A. Fogo, F. Niimura, I. Ichikawa, B. L. M. Hogan, and T. Inagami.** Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature Lond.* 377: 748–750, 1995.
31. **Izumo, S., B. Nadal-Ginard, and V. Mahdavi.** Proto-oncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc. Natl. Acad. Sci. USA* 85: 339–343, 1988.
32. **Johns, D. W., M. J. Peach, R. A. Gomez, T. Inagami, and R. M. Carey.** Angiotensin II regulates renin gene expression. *Am. J. Physiol.* 259 (*Renal Fluid Electrolyte Physiol.* 28): F882–F887, 1990.
33. **Kambayashi, Y., S. Bardhan, K. Takahashi, S. Tsuzuki, H. Inui, T. Hamakubo, and T. Inagami.** Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J. Biol. Chem.* 268: 24543–24546, 1993.
34. **Kim, S., M. Hosoi, F. Ikemoto, K. Murakami, Y. Ishizuka, and K. Yamamoto.** Conversion to renin of exogenously administered recombinant human prorenin in liver and kidney of monkeys. *Am. J. Physiol.* 258 (*Endocrinol. Metab.* 21): E451–E458, 1990.
35. **Krege, J. H., S. W. M. John, L. L. Langenbach, J. B. Hodgin, J. R. Hagaman, E. S. Bachman, J. C. Jennette, D. A. O'Brien, and O. Smithies.** Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature Lond.* 375: 146–148, 1995.
36. **Kumar, R. S., J. Kusari, S. N. Roy, R. L. Soffer, and G. C. Sen.** Structure of testicular angiotensin-converting enzyme. *J. Biol. Chem.* 264: 16754–16758, 1989.
37. **Kurtz, T. W., L. Simonet, P. M. Kabra, S. Wolfe, L. Chan, and B. L. Hjelle.** Cosegregation of the renin allele of the spontaneously hypertensive rat with an increase in blood pressure. *J. Clin. Invest.* 85: 1328–1332, 1990.
38. **Lafayette, R. A., G. Mayer, S. K. Park, and T. W. Meyer.** Angiotensin II receptor blockade limits glomerular injury in rats with reduced renal mass. *J. Clin. Invest.* 90: 766–771, 1992.
39. **Lee, M., M. Böhm, S. Kim, S. Bachmann, J. Bachmann, M. Bader, and D. Ganten.** Differential gene expression of renin and angiotensinogen in the TGR(mREN2)27 transgenic rat. *Hypertension Dallas* 25: 570–580, 1995.
40. **Lee, M., Y. Zhao, J. Peters, D. Ganten, F. Zimmermann, U. Ganten, S. Bachmann, M. Bader, and J. J. Mullins.** Preparation and analysis of transgenic rats expressing the mouse Ren-2 gene. *J. Vasc. Med. Biol.* 3: 50–54, 1991.
41. **Lemmer, B., A. Mattes, M. Böhm, and D. Ganten.** Circadian blood pressure variation in transgenic hypertensive rats. *Hypertension Dallas* 22: 97–101, 1993.
42. **Levens, N. R., M. J. Peach, and R. M. Carey.** Role of the intrarenal renin-angiotensin system in the control of renal function. *Circ. Res.* 48: 157–167, 1981.
43. **Lifton, R. P., R. G. Dluhy, M. Powers, G. M. Rich, S. Cook, S. Ulick, and J.-M. Lalouel.** A chimaeric 11β-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. *Nature Lond.* 355: 262–265, 1992.
44. **Lindpaintner, K.** Genetic linkage analysis in hypertension: principles and practice. *J. Hypertens.* 10: 121–124, 1992.
45. **Lindpaintner, K., and D. Ganten.** The cardiac renin-angiotensin system—an appraisal of present experimental and clinical evidence. *Circ. Res.* 68: 905–921, 1991.
46. **Mitchell, K. D., and J. J. Mullins.** ANG II dependence of tubuloglomerular feedback responsiveness in hypertensive *ren*-2 transgenic rats. *Am. J. Physiol.* 268 (*Renal Fluid Electrolyte Physiol.* 37): F821–F828, 1995.
47. **Moriguchi, A., K. B. Brosnihan, H. Kumagai, D. Ganten, and C. M. Ferrario.** Mechanisms of hypertension in transgenic rats expressing the mouse Ren-2 gene. *Am. J. Physiol.* 266 (*Regulatory Integrative Comp. Physiol.* 35): R1273–R1279, 1994.
48. **Moriguchi, A., C. M. Ferrario, K. B. Brosnihan, D. Ganten, and M. Morris.** Differential regulation of central vasopressin in transgenic rats harboring the mouse Ren-2 gene. *Am. J. Physiol.* 267 (*Regulatory Integrative Comp. Physiol.* 36): R786–R791, 1994.
49. **Moriguchi, A., E. A. Tallant, K. Matsumura, T. M. Reilly, H. Walton, D. Ganten, and C. M. Ferrario.** Opposing actions of angiotensin-(1—7) and angiotensin II in the brain of transgenic hypertensive rats. *Hypertension Dallas* 25: 1260–1265, 1995.
50. **Müller, J.** *Monographs on Endocrinology, Regulation of Aldosterone Biosynthesis: Physiological and Clinical Aspects.* Berlin: Springer Verlag, vol. 29, 1988.
51. **Mullins, J. J., J. Peters, and D. Ganten.** Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature Lond.* 344: 541–544, 1990.
52. **Mullins, J. J., C. D. Sigmund, C. Kane-Haas, R. A. McGowan, and K. W. Gross.** Expression of the DBA/2J Ren-2 gene in the adrenal gland of transgenic mice. *EMBO J.* 8: 4065–4072, 1989.
53. **Naftilan, A. J., R. E. Pratt, C. S. Eldridge, H. L. Lin, and V. J. Dzau.** Angiotensin II induces c-fos expression in smooth muscle via transcriptional control. *Hypertension Dallas* 13: 706–711, 1989.
54. **Nielsen, A. H., and K. Poulsen.** Is prorenin of physiological and clinical significance? *J. Hypertens.* 6: 949–958, 1988.
55. **Oliver, W. J., and F. Gross.** Unique specificity of mouse angiotensinogen to homologous renin. *Proc. Soc. Exp. Biol. Med.* 122: 923–926, 1966.
56. **Onoyama, K., T. Omae, and T. Inagami.** Tissue edema and arterial lesions produced by pure submaxillary gland renin of mouse. *Jpn. Heart J.* 19: 522–530, 1978.
57. **Paul, M., Y. M. Pinto, H. Schunkert, D. Ganten, and M. Böhm.** Activation of the renin-angiotensin system in heart failure and hypertrophy—studies in human hearts and transgenic rats. *Eur. Heart J.* 15: 63–67, 1994.
58. **Paul, M., J. Wagner, and V. J. Dzau.** Gene expression of the components of the renin-angiotensin system in human tissues: quantitative analysis by the polymerase chain reaction. *J. Clin. Invest.* 91: 2058–2064, 1993.
59. **Peters, J., K. Münter, M. Bader, E. Hackenthal, J. J. Mullins, and D. Ganten.** Increased adrenal renin in transgenic hypertensive rats, TGR(mREN2)27, and its regulation by cAMP, angiotensin II and calcium. *J. Clin. Invest.* 91: 742–747, 1993.
60. **Phillips, M. I.** Functions of angiotensin in the central nervous system. *Annu. Rev. Physiol.* 49: 413–435, 1987.
61. **Rapp, J. P.** Genetics of experimental and human hypertension. In: *Hypertension*, edited by J. Genest, O. Kuchel, P. Hamet, and M. Cantin. New York: McGraw-Hill, 1983, p. 582–598.
62. **Rapp, J. P., S. M. Wang, and H. Dene.** Mutations in the renin gene of Dahl rats. *Hypertension Dallas* 12: 339–342, 1988.
63. **Rong, P., J. L. Berka, D. J. Kelly, D. Alcorn, and S. L. Skinner.** Renin processing and secretion in adrenal and retina of transgenic (mREN2)27 rats. *Kidney Int.* 46: 1583–1587, 1994.

64. **Rubattu, S., I. Enea, D. Ganten, D. Salvatore, G. Condorelli, G. Condorelli, R. Russo, M. Romano, B. Gigante, B. Trimarco, and M. Volpe.** Enhanced adrenal renin and aldosterone biosynthesis during sodium restriction in TGR (mREN2)27. *Am. J. Physiol.* 267 (*Endocrinol. Metab.* 30): E515–E520, 1994.
65. **Sadoshima, J., Y. Xu, H. S. Slayter, and S. Izumo.** Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* 75: 977–984, 1993.
66. **Sander, M., M. Bader, B. Djavidani, C. Maser-Gluth, P. Vecsei, J. Mullins, D. Ganten, and J. Peters.** The role of the adrenal gland in the hypertensive transgenic rats TGR(mREN2)27. *Endocrinology* 131: 807–814, 1992.
67. **Sander, M., D. Ganten, and S. H. Mellon.** Role of adrenal renin in the regulation of adrenal steroidogenesis by corticotropin. *Proc. Natl. Acad. Sci. USA* 91: 148–152, 1994.
68. **Schunkert, H., L. Jahn, S. Izumo, C. S. Apstein, and B. H. Lorell.** Localization and regulation of c-fos and c-jun protooncogene induction by systolic wall stress in normal and hypertrophied rat hearts. *Proc. Natl. Acad. Sci. USA* 88: 11480–11484, 1991.
69. **Senanayake, P., A. Moriguchi, H. Kumagai, D. Ganten, C. M. Ferrario, and K. B. Brosnihan.** Increased expression of angiotensin peptides in the brain of transgenic hypertensive rats. *Peptides* 15: 919–926, 1994.
70. **Shimkets, R. A., D. G. Warnock, C. M. Bositis, C. Nelson-Williams, J. H. Hansson, M. Schambelan, J. R. Gill, S. Ulick, R. V. Milora, J. W. Findling, C. M. Canessa, B. C. Rossier, and R. P. Lifton.** Liddles syndrome: heritable human hypertension caused by mutations in the β-subunit of the epithelial sodium channel. *Cell* 79: 407–414, 1994.
71. **Sigmund, C. D., and K. W. Gross.** Structure, expression, and regulation of the murine renin genes. *Hypertension Dallas* 18: 446–457, 1991.
72. **Sigmund, C. D., C. A. Jones, J. R. Fabian, J. J. Mullins, and K. W. Gross.** Tissue and cell specific expression of a renin promoter-T-antigen-reporter gene construct in transgenic mice. *Biochem. Biophys. Res. Commun.* 170: 344–350, 1990.
73. **Skinner, S. L., R. L. Thatcher, J. A. Whitworth, and J. D. Horowitz.** Extraction of plasma prorenin by human heart. *Lancet* 1: 995–997, 1986.
74. **Smithies, O., and M. Nobuyo.** Gene targeting approaches to complex genetic diseases: arteriosclerosis and essential hypertension. *Proc. Natl. Acad. Sci. USA* 92: 5266–5272, 1995.
75. **Springate, J. E., L. G. Feld, and D. Ganten.** Renal function in hypertensive rats transgenic for mouse renin gene. *Am. J. Physiol.* 266 (*Renal Fluid Electrolyte Physiol.* 35): F731–F737, 1994.
76. **Susuki, J., H. Matsubara, M. Urakami, and M. Inada.** Rat angiotensin II (type-1A) receptor mRNA regulation and subtype expression in myocardial growth and hypertrophy. *Circ. Res.* 73: 439–447, 1993.
77. **Tang, S. S., and V. J. Dzau.** Partial purification of a prorenin activating enzyme in the aortic wall. *Clin. Exp. Hypertens. A* 10: 1251–1257, 1988.
78. **TawÆk-Schlieper, H., M. Moll, B. Schmid, R. H. Schwinger, M. Paul, D. Ganten, and M. Bo hm.** Alterations of cardiac α- and β-adrenoreceptors and inotropic responsiveness in hypertensive transgenic rats harboring the mouse renin gene (TGR(mREN2)27). *Clin. Exp. Hypertens.* 17: 631–648, 1995.
79. **Thybo, N. K., N. Korsgaard, and M. J. Mulvany.** Morphology and function of mesenteric resistance arteries in transgenic rats with low-renin hypertension. *J. Hypertens.* 10: 1191–1196, 1992.
80. **Tokita, Y., R. Franco-Saenz, P. J. Mulrow, and D. Ganten.** Effects of nephrectomy and adrenalectomy on the renin-angiotensin system of transgenic rats TGR(mRen2)27. *Endocrinology* 134: 253–257, 1994.
81. **Tokita, Y., R. Franco-Saenz, E. M. Reimann, and P. J. Mulrow.** Hypertension in the transgenic rat TGR(mRen2)27 may be due to enhanced kinetics of the reaction between mouse renin and rat angiotensinogen. *Hypertension Dallas* 23: 422–427, 1994.
82. **Tronik, D., and F. Rougeon.** Thyroxine and testosterone transcriptionally regulate renin gene expression in the submaxillary gland of normal and transgenic mice carrying extra copies of the Ren-2 gene. *FEBS Lett.* 234: 336–340, 1988.
83. **Tschudi, M. R., G. Noll, U. Arnet, D. Novosel, D. Ganten, and T. F. Lu scher.** Alterations in coronary artery vascular reactivity of hypertensive Ren-2 transgenic rats. *Circulation* 89: 2780–2786, 1994.
84. **Unger, T., B. Schu ll, D. Hu bner, T. Yukimura, R. E. Lang, W. Rascher, and D. Ganten.** Plasma-converting enzyme activity does not reflect effectiveness of oral treatment with captopril. *Eur. J. Pharmacol.* 72: 255–259, 1981.
85. **Volpe, M., S. Rubattu, B. Gigante, D. Ganten, A. Porcellini, R. Russo, M. Romano, I. Enea, M. Lee, and B. Trimarco.** Regulation of aldosterone biosynthesis by adrenal renin is mediated through AT1 receptors in renin transgenic rats. *Circ. Res.* 77: 73–79, 1995.
86. **Waeber, B., H. R. Brunner, D. B. Brunner, A. L. Curtet, G. A. Turini, and H. Gavras.** Discrepancy between antihypertensive effect and angiotensin converting enzyme inhibition by captopril. *Hypertension Dallas* 2: 236–242, 1980.
87. **Wagner, D., R. Metzger, M. Paul, G. Ludwig, F. Suzuki, S. Takahashi, K. Murakami, and D. Ganten.** Androgen dependence and tissue specificity of renin messenger RNA expression in mice. *J. Hypertens.* 8: 45–52, 1990.
88. **Whitworth, C. E., S. Fleming, A. D. Cumming, J. J. Morton, N. J. T. Burns, B. C. Williams, and J. J. Mullins.** Spontaneous development of malignant phase hypertension in transgenic Ren-2 rats. *Kidney Int.* 46: 1528–1532, 1994.
89. **Wu, J. N., and K. H. Berecek.** Prevention of genetic hypertension by early treatment of spontaneously hypertensive rats with the angiotensin converting enzyme inhibitor captopril. *Hypertension Dallas* 22: 139–146, 1993.
90. **Yamaguchi, T., Z. Naito, G. D. Stoner, R. Franco-Saenz, and P. J. Mulrow.** Role of the adrenal renin-angiotensin system on adrenocorticotropic hormone- and potassium-stimulated aldosterone production by rat adrenal glomerulosa cells in monolayer culture. *Hypertension Dallas* 16: 635–641, 1990.
91. **Yamaguchi, T., Y. Tokita, R. Franco-Saenz, P. J. Mulrow, J. Peters, and D. Ganten.** Zonal distribution and regulation of adrenal renin in a transgenic model of hypertension in the rat. *Endocrinology* 131: 1955–1962, 1992.
92. **Zhao, Y., M. Bader, R. Kreutz, M. Fernandez-Alfonso, F. Zimmermann, U. Ganten, R. Metzger, D. Ganten, J. J. Mullins, and J. Peters.** Ontogenetic regulation of mouse *Ren-2*^{d} renin gene in transgenic hypertensive rats, TGR(mREN2)27. *Am. J. Physiol.* 265 (*Endocrinol. Metab.* 28): E699–E707, 1993.

ADVANCES IN PHYSIOLOGY EDUCATION
See Part 3, S1–S93

JUNE 1996
Volume 270/Number 6
Part 1 of Three Parts
ISSN 0002-9513

American Journal of Physiology

PUBLISHED BY
THE AMERICAN PHYSIOLOGICAL SOCIETY

EXHIBIT D

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11153–11157, November 1994
Applied Biological Sciences

Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection

WILLIAM L. FODOR*†, BARRY L. WILLIAMS*, LOUIS A. MATIS*, JOSEPH A. MADRI‡, SCOTT A. ROLLINS*, JAMES W. KNIGHT§, WILLIAM VELANDER¶, AND STEPHEN P. SQUINTO*†

*Alexion Pharmaceuticals, Inc., 25 Science Park, New Haven, CT 06511; ‡ Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, CT 06510; and Departments of §Animal Sciences and ¶Chemical Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

Communicated by George E. Seidel, Jr., June 23, 1994

ABSTRACT **The serious shortage of human organs available for transplantation has engendered a heightened interest in the use of animal organs (xenografts) for transplantation. However, the major barrier to successful discordant xenogeneic organ transplantation is the phenomenon of hyperacute rejection. Hyperacute rejection results from the deposition of high-titer preformed antibodies that activate serum complement on the luminal surface of the vascular endothelium, leading to vessel occlusion and graft failure within minutes to hours. Although endogenous membrane-associated complement inhibitors normally protect endothelial cells from autologous complement, they are species restricted and thus confer limited resistance to activated xenogeneic complement. To address the pathogenesis of hyperacute rejection in xenotransplantation, transgenic mice and a transgenic pig were engineered to express the human terminal complement inhibitor hCD59. High-level cell surface expression of hCD59 was achieved in a variety of murine and porcine cell types, most importantly on both large vessel and capillary endothelium. hCD59-expressing porcine cells were significantly resistant to challenge with high-titer anti-porcine antibody and human complement. These experiments demonstrate a strategy for developing a pig-to-primate xenogeneic transplantation model to test whether the expression of a human complement inhibitor in transgenic pigs could render xenogeneic organs resistant to hyperacute rejection.**

The lack of effective therapies aimed at eliminating antibody- and complement-mediated hyperacute rejection presents a major barrier to the successful transplantation of discordant animal organs into human recipients (1–6) and has precluded the development of animal models aimed at evaluating the *in vivo* cellular immune response to discordant xenografts. Old World primates, including humans, have high levels of preexisting circulating natural antibodies that predominantly recognize carbohydrate determinants expressed on the surface of xenogeneic cells from discordant species (2–6). Recent evidence indicates that most of these antibodies react with the carbohydrate epitope, Gal(α1-3)Gal (7), an epitope absent from Old World primates because of a lack of the functional α-1,3-galactosyltransferase enzyme (8). Therefore, after transplantation of a vascularized xenogeneic donor organ into a primate recipient, the massive inflammatory response that ensues from natural antibody activation of the classical complement cascade leads to activation and destruction of the vascular endothelial cells and ultimately of the donor organ within minutes to hours after revascularization (2–6). Endogenously expressed membrane-associated complement regulatory proteins normally protect endothelial cells from autologous complement. However, the activity of these complement inhibitors is species restricted. This property makes them relatively ineffective at inhibiting xenogeneic serum complement (9, 10). The demonstration that a human complement inhibitor could protect a xenogeneic cell from human complement-mediated lysis showed that it was possible to inhibit human anti-porcine hyperacute rejection in *in vitro* models (11).

The strategy used to address the pathogenesis of hyperacute rejection in the porcine-to-primate xenotransplantation model was to produce transgenic swine expressing high levels of the human terminal complement inhibitor hCD59. hCD59 is an 18- to 20-kDa glycosyl-phosphatidylinositol-anchored cell surface glycoprotein that is expressed in a variety of tissues of both hematopoietic and nonhematopoietic lineage and functions to inhibit formation of the membrane attack complex by binding to membrane C5b-8 and C5b-9 (9, 10). Stable expression of hCD59 on xenogeneic cells *in vitro* protected the cells from human complement-mediated cell lysis (12–14) and the level of protection was directly proportional to the number of molecules of hCD59 expressed on the surface of the xenogeneic cell (14). Importantly, hCD59-expressing porcine aortic endothelial cells were resistant not only to cell lysis but also to complement-mediated formation of a procoagulant surface when challenged with either human or baboon serum (15). Taken together, these results indicated that high-level expression of hCD59 could provide porcine tissue with significant protection from human serum complement in a xenotransplantation setting. Therefore, hCD59 was chosen as a candidate molecule for production of transgenic swine resistant to human complement. In this report, we demonstrate the successful production of a transgenic pig expressing high levels of hCD59 that protect the pig cells from human complement-mediated cell lysis.

MATERIALS AND METHODS

***H2K^b*–hCD59 DNA Construct, Purification, and Microinjection.** A hCD59 cDNA was directionally cloned into exon 1 of the murine *H2K^b*-gene 12 nucleotides downstream of the transcriptional start site. Briefly, the hCD59 cDNA fragment was excised from a hCD59-pcDNAI-Amp (pcDNAI-Amp; Invitrogen) expression plasmid by digestion with *Hin*dIII, followed by enzymatically filling in the 5′ 4-nucleotide overhang with T4 DNA polymerase and dNTPs. Subsequently,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; PBMCs, peripheral blood mononuclear cells; mAb, monoclonal antibody; PHA, phytohemagglutinin; FITC, fluorescein isothiocyanate; hrTNF-α, human recombinant tumor necrosis factor α; IFN, interferon.

†To whom reprint requests should be addressed.

the DNA was digested with *Not* I at the 3′ end of the multiple cloning site of the vector to yield a 452-bp cDNA fragment. The 9.0-kbp *Eco*RI $H2K^b$ genomic restriction fragment (16) cloned into pGEM7Z (Promega) was digested with *Nru* I and *Not* I, resulting in the removal of 51 nucleotides from the $H2K^b$ gene including the ATG start codon. The hCD59 cDNA was then directionally ligated into the $H2K^b$ gene in the pGEM7Z vector.

Purification of the $H2K^b$–hCD59 DNA for embryo injection was accomplished by digesting the plasmid with *Xho* I to remove the vector sequences followed by agarose gel electrophoresis, electroelution, and Elutip purification (Schleicher & Schull). Transgenic mice were produced by pronuclear microinjection of murine ova as described (17). Ten of 60 offspring were identified as transgenic founder animals by DNA slot blot hybridization (18) (data not shown). Transgenic swine were generated by porcine embryo injection (19). A total of 18 piglets were analyzed by DNA slot blot analysis of genomic DNA (18). One founder animal, $H2K^b$–hCD59 153-2, contained 10–20 copies of the $H2K^b$–hCD59 DNA. Two additional founder animals, $H2K^b$–hCD59 152-1 and $H2K^b$–hCD59 152-2, contained ≈1 copy of the $H2K^b$–hCD59 DNA and exhibited no expression or very low and inconsistent levels of expression in peripheral blood mononuclear cells (PBMCs) (data not shown). These animals were not analyzed further.

Cell Culture, Immunofluorescence, and Immunohistochemistry. PBMCs from transgenic and negative littermate control pigs were purified from whole blood by Ficoll gradient centrifugation (ref. 20, pp. 7.1.1–7.1.2). Adherent monocytic mononuclear cells were cultured in Dulbecco's modified Eagle's medium/15% fetal bovine serum. PBMCs from transgenic mice and negative littermate control animals were purified from whole blood by ACK lysis (Biofluids, Rockville, MD). Indirect immunofluorescence of porcine PBMCs was performed with the anti-hCD59 mouse monoclonal antibody (mAb) MEM-43 (Biodesign International, Kennebunkport, ME) and with the anti-swine leukocyte antigen (SLA) class I mAb PT85A (VMRD, Pullman, WA). Indirect immunofluorescence of murine PBMCs was performed with polyclonal antisera specific for hCD59 (P. Sims, Blood Research Institute, Milwaukee). Goat anti-rabbit IgG (polyclonal sera; Zymed) or goat anti-mouse IgG (monoclonal sera; Zymed) fluorescein isothiocyanate (FITC)-conjugated antisera were used to detect specific antibody binding to the cell surface. Cell surface expression was then measured by flow cytometry on a Becton Dickinson FACSort.

The cytokine inducibility of $H2K^b$–hCD59 and the endogenous porcine SLA class I molecule was tested on adherent peripheral blood monocytes. Briefly, porcine cytokine-conditioned medium supernatants were produced from control pig PBMCs. PBMCs harvested from a control pig were stimulated with phytohemagglutinin (PHA; 5 μg/ml) for 48 h. PHA-conditioned media were collected and treated with 10 mM methyl α-mannoside and filter sterilized. Human recombinant tumor necrosis factor α (hrTNF-α; Collaborative Biomedical Products, Bedford, MA) was used at 500 units/ml. Adherent peripheral blood monocytes were then treated with medium alone, 50% PHA-conditioned medium (diluted 1:1 with complete medium), 50% PHA-conditioned medium/hrTNF-α, or hrTNF-α for 24 h. Cytokine-induced expression of hCD59 and SLA class I was detected by immunofluorescence and fluorescence-activated cell sorter analysis as described above.

Immunohistochemistry was performed on fresh frozen sections embedded in Tissue-Tek OCT compound (Miles). Tissue sections (5–10 μm) were processed as described (ref. 20, pp. 5.8.1–5.8.2). Sections that were double stained were processed simultaneously with the mouse anti-hCD59 mAb, MEM-43 (20 μg/ml), and the anti-type IV collagen rabbit polyclonal antiserum (21) (1:50 dilution). Fluorochrome-conjugated goat anti-mouse IgG and goat anti-rabbit IgG antisera were used to detect specific antibody interactions with the hCD59 antigen (goat anti-mouse rhodamine; AMAC, Westbrook, ME) and type IV collagen antigen (goat anti-rabbit FITC; Zymed).

Complement-Mediated Dye Release Assays. PBMCs or peripheral blood adherent cells were labeled with the intracellular dye Calcein AM (Molecular Probes). The cells were subsequently incubated with anti-porcine blood cell IgG (2 mg/ml) (Intercell Technologies, Hopewell, NJ) followed by incubation in increasing concentrations of human whole serum (Sigma) at 37°C for 30 min. Dye released from the cells was determined by flow cytometry on a Becton Dickinson FACSort. The C5b-9-specific dye release was calculated as percentage of total, correcting for nonspecific dye release and background fluorescence measured on identically matched controls without the addition of serum. Antibody blocking experiments were performed by the complement-mediated dye release assay as described above with the following exceptions. The cells were incubated in 20% C8-deficient serum (C8d; Quidel, San Diego) at 37°C for 30 min after anti-porcine blood cell antibody activation. The cells were then incubated with hCD59 polyclonal antiserum (100 μg/ml) or anti-SLA class I antiserum PT85A (100 μg/ml). Purified human C8 (Quidel) and C9 (Quidel) complement components were then added in increasing concentrations and incubated at 37°C for 30 min. Dye released from the cells was detected by flow cytometry on a Becton Dickinson FACSort as described above.

RESULTS

Transgenic Expression. To achieve expression of the transgene-encoded hCD59 we engineered a murine major histocompatibility complex (MHC) class I gene, $H2K^b$ (16), to control the expression of a hCD59 cDNA, $H2K^b$–hCD59 (Fig. 1). The MHC class I gene is ubiquitously expressed on most somatic cells and, most importantly, is a predominant endothelial cell surface antigen (22, 24). In addition, the MHC class I promoter contains cis-acting regulatory elements that bind cytokine-inducible trans-acting factors, resulting in up-regulation of the class I gene upon stimulation with interferon (IFN)-α/β, IFN-γ, and TNF-α (22–25). A hCD59 cDNA was cloned into exon I of $H2K^b$ and results in a transcript that initiates at the $H2K^b$ transcriptional start site and proceeds through both the cDNA insert and the entire transcriptional unit of the $H2K^b$ gene. Translation initiates at the ATG codon of the inserted cDNA and terminates at the cDNA stop codon. The rest of the $H2K^b$ gene remains untranslated and functions only in RNA processing, providing the cDNA with a genomic structure that contains all the regulatory elements required for $H2K^b$ expression (22–25).

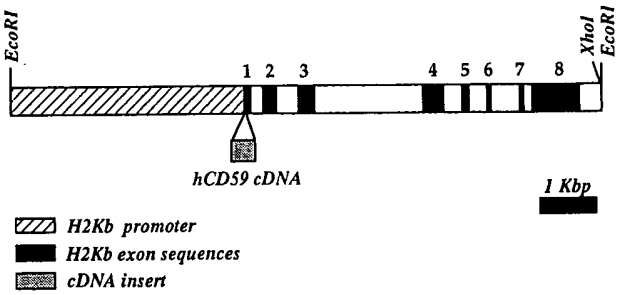


FIG. 1. $H2K^b$ genomic cassette. A linear representation of the hybrid gene construct detailing the exon–intron structure of $H2K^b$ and the insertion of the hCD59 cDNA into exon 1.

The efficacy of the *H2K*^{b}–hCD59 genomic expression construct in directing cell surface expression of hCD59 in various tissues was tested in transgenic mice and pigs. Initial analysis demonstrated that the *H2K*^{b}–hCD59 genomic construct directed the expression of hCD59 on the surface of PBMCs in several founder transgenic mice and transgenic pig 153-2 (Fig. 2 *A* and *B*, respectively). Importantly, expression of hCD59 on the surface of the porcine mononuclear cells paralleled that of SLA class I (Fig. 2*B*). The comparable expression of hCD59 to SLA class I indicated that the murine

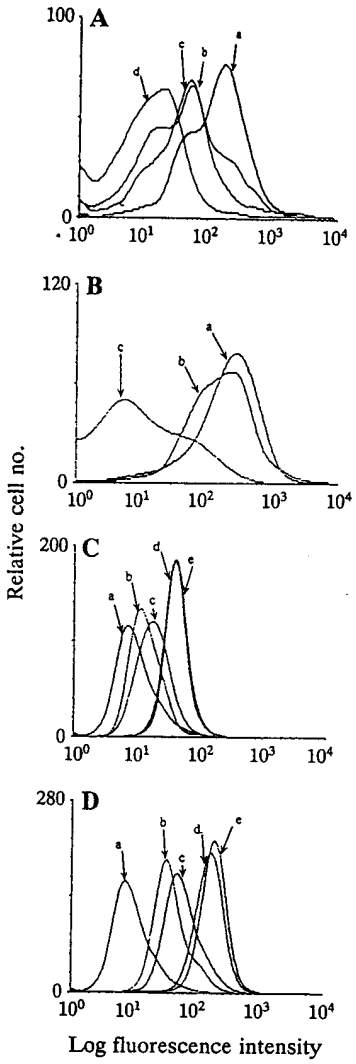


FIG. 2. Cell surface expression of hCD59 in transgenic mice and a transgenic pig. (*A*) Expression of hCD59 on murine PBMCs detected in transgenic mice *H2K*^{b}–CD59-11 (curve a), *H2K*^{b}–CD59-23 (curve b), *H2K*^{b}–CD59-21 (curve c), and a negative littermate control (curve d). (*B*) Cell surface expression of hCD59 and SLA class I detected on porcine PBMCs. Curve a, hCD59 expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve b, SLA class I expression in transgenic pig *H2K*^{b}–hCD59 153-2; curve c, negative littermate control PBMCs incubated with the hCD59 mAb. (*C*) Cytokine-induced cell surface expression of hCD59 on cultured adherant PMBCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antisera (curve a); hCD59 expression on uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e). (*D*) Cytokine-induced cell surface expression of SLA class I on cultured adherent PBMCs from pig *H2K*^{b}–hCD59 153-2; goat-anti-mouse FITC control antiserum (curve a); uninduced cells (curve b); hrTNF-α (curve c); PHA conditioned medium (curve d); PHA conditioned medium + hrTNF-α (curve e).

H2K^b–hCD59 chimeric gene was constitutively regulated, similar to the endogenous porcine SLA class I molecules. To establish whether the *H2K*^{b}–hCD59 chimeric gene exhibited cytokine inducibility comparable to the endogenous SLA class I gene, we cultured adherent monocytic PBMCs. Interestingly, after prolonged culture, these monocytes had downregulated cell surface expression of both SLA class I as well as the hCD59 transgene-encoded protein (compare Fig. 2*B*, curve b, to Fig. 2*D*, curve b for class I and Fig. 2*B*, curve a, to Fig. 2*C*, curve b, for hCD59). Treatment of the transgenic porcine cells with PHA-induced cytokine-conditioned medium, with hrTNF-α, or with a combination of the treatments resulted in an increase in hCD59 expression (Fig. 2*C*) as well as an increase in SLA class I expression (Fig. 2*D*).

We next examined hCD59 expression on the endothelium of vascularized organs. Immunohistochemical analyses were performed on fresh-frozen tissue sections derived from hCD59 transgenic mice and pigs as well as from nontransgenic littermates. Phase-contrast micrographs illustrating the structure of mouse myocardium are shown in Fig. 3 *A* and *D*. Tissue sections from three founder mice were analyzed for hCD59 expression. Mouse hearts were incubated with anti-collagen type IV polyclonal antisera to detect basement membrane structures underlying the endocardium as well as intramyocardial capillary endothelia (21). Fig. 3 *B* and *E*, respectively, confirmed equivalent collagen staining in the negative littermate control and a representative hCD59 transgenic mouse, *H2K*^{b}–hCD59-8. In contrast, staining with a mAb specific for hCD59 revealed intense cell surface expression on endothelial cells in the heart of transgenic mouse *H2K*^{b}–hCD59-8 (Fig. 3*F*) and an absence of hCD59 expression in the negative littermate control (Fig. 3*C*). Fig. 3*F* dramatically highlights the expression of hCD59 on vascular structures and clearly shows high-level expression of hCD59 on the endocardium in the ventricular chamber. Abundant hCD59 was also detected on capillary vessels within the myocardium (Fig. 3*F*). All three founder transgenic mice analyzed revealed hCD59 staining on the endocardium and capillary endothelium. To evaluate vascularized structures in the transgenic pig without having to sacrifice the founder animal, tail sections were prepared and analyzed by immunohistochemistry as described for the mice. Phase-contrast micrographs illustrate the morphological structure of a tail artery from a negative control pig (Fig. 4*A*) and a tail artery

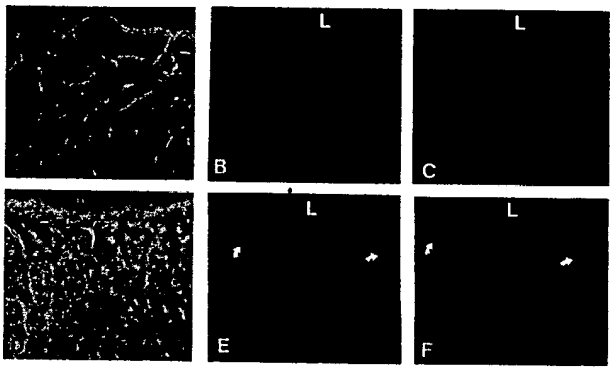


FIG. 3. Double-label immunofluorescence microscopy of hCD59 and type IV collagen on murine heart tissue from a *H2K*^{b}–hCD59 transgenic mouse and a negative littermate control. Phase-contrast micrographs of murine ventricular myocardium (*A* and *D*). L, lumen of the left ventricle lined by endothelial cells. (*B* and *E*) Immunofluorescence micrographs detecting type IV collagen (fluorescein) of the same myocardial sections illustrating basement membrane structures underlying the endocardium. Immunofluorescence micrographs (rhodamine) of the same myocardial sections detecting hCD59 in a negative littermate control (*C*) and *H2K*^{b}–hCD59-8 (*F*). (×400.) (Bar = 25 μm.)

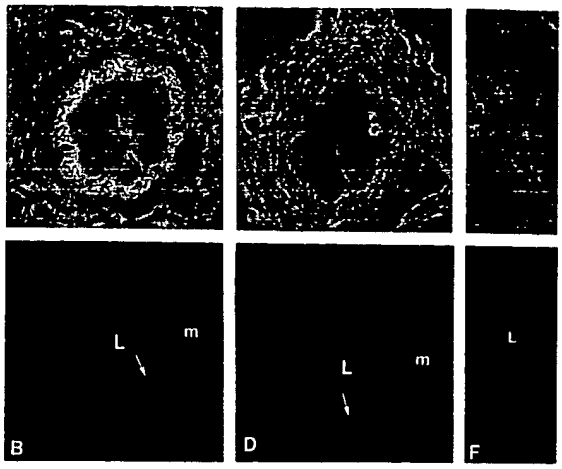


FIG. 4. Immunofluorescence microscopy of hCD59 on swine tail sections from pig $H2K^b$–hCD59 153-2 and a negative littermate control. (*A*) Phase-contrast micrograph of a dermal artery from the negative littermate illustrating the lumen (L), the endothelial cell layer (arrow), and the tunica media (m). (*B*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *A*, illustrating the lumen, the endothelial cell layer, and the tunica media. (*C*) Phase-contrast micrograph of a dermal artery from pig $H2K^b$–hCD59 153-2, illustrating the lumen, the endothelial cell layer, and the tunica media. (*D*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *C*, illustrating the lumen, the endothelial cell layer, and the medial smooth muscle cells (m). (*E*) Phase-contrast micrograph of a dermal microvessel from pig $H2K^b$–hCD59 153-2, illustrating the lumen and the vessel wall. (*F*) Immunofluorescence micrograph (rhodamine) of the same section pictured in *E*, illustrating the lumen, and an abundance of hCD59 expression. (×400.) (Bar = 25 μm.)

and small vessel from the transgenic founder pig 153-2 (Fig. 4 *C* and *E*, respectively). High-level hCD59 expression was observed on a variety of tissue and cell types, including fibroblasts, epithelial cells, vascular endothelial cells, and smooth muscle cells within the tail section of the transgenic pig (Fig. 4 *D* and *F*) but not in the negative littermate (Fig. 4*B*). Not all tissue in the transgenic pig tail section revealed hCD59 staining; however, tissues such as striated muscle are known to express very low levels of the class I antigen and therefore would not be expected to express the class I-regulated hCD59 transgene (24). These analyses confirmed that the $H2K^b$–hCD59 genomic construct directed expression of hCD59 to a variety of cells and tissues in transgenic pig 153-2 and, most importantly, to the surface of vascular endothelial cells.

Complement Resistance. To determine whether the high levels of transgene expression observed on the transgenic pig cells conferred significant protection from human complement-mediated attack, functional analyses were performed on hCD59-expressing porcine PBMCs collected from transgenic pig 153-2 and a nontransgenic littermate control. The data clearly demonstrated that hCD59-expressing porcine cells, but not cells from a nontransgenic littermate, significantly resisted human complement-mediated lysis (Fig. 5*A*). The percentage dye released from hCD59 protected cell was ≈5-fold less when compared with the amount of dye released from negative littermate control cells. To confirm that the protection observed in PBMCs was due specifically to hCD59 expression, antibody blocking experiments were performed. As shown in Fig. 5*B*, the anti-hCD59 polyclonal antisera blocked the hCD59-mediated protection, resulting in an increased susceptibility of the porcine cells to human complement-mediated cell lysis. In contrast, the control antibody had no effect.

To evaluate whether the degree of protection of porcine cells from human complement attack was a function of the

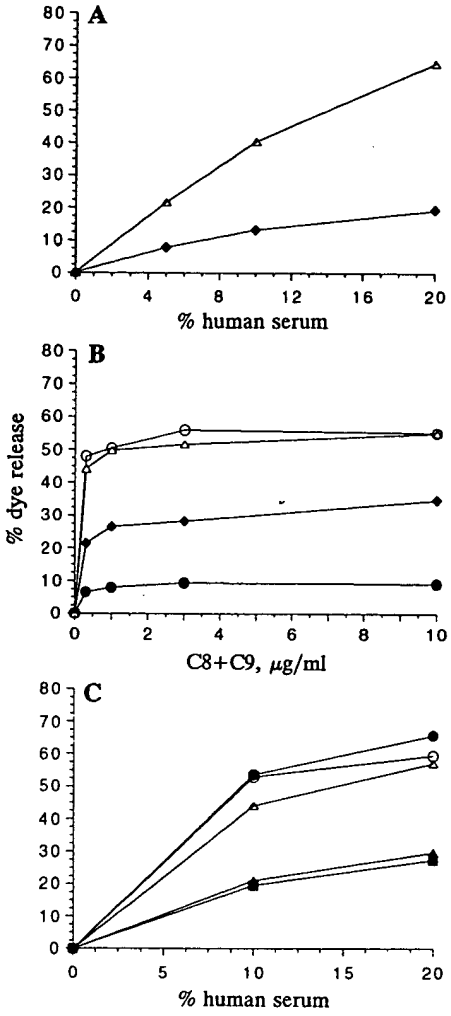


FIG. 5. Complement-mediated dye release assays on porcine PBMCs and cultured peripheral blood adherent cells. (*A*) Dye release assay performed on porcine PBMCs (◆), transgenic pig $H2K^b$–hCD59 153-2; (△), negative littermate control. (*B*) Dye release assay performed on PBMCs from transgenic pig $H2K^b$–hCD59 153-2 incubated in the presence of anti-hCD59 polyclonal antiserum (◆); control class I antibody PT85A (●); negative littermate control PBMCs incubated in the presence of anti-hCD59 polyclonal antiserum (△); control class I antibody PT85A (○). (*C*) Complement-mediated dye release assays on porcine peripheral blood adherent cells from pig $H2K^b$–hCD59 153-2; uninduced cells (△), PHA supernatants (■), PHA supernatants + hrTNF-α (▲), hrTNF-α (●), and control negative littermate cultured peripheral blood adherent cells (○).

level of hCD59 expressed on the cell surface, experiments were performed on the cultured monocyte lines derived from the $H2K^b$–hCD59 153-2 transgenic pig, which showed increased cell surface expression in response to cytokine treatments (see Fig. 2). Significantly, these monocytes demonstrated increased susceptibility to human complement-mediated lysis, consistent with the loss of hCD59 expression (Fig. 5*C*). As previously shown, culture of these cells in the presence of cytokines known to induce the MHC class I promoter—i.e., IFN-γ and TNF-α—upregulated hCD59 expression (Fig. 2*C*). Importantly, upregulating hCD59 expression restored their complement-resistant phenotype (Fig. 5*C*). These results confirm that the level of transgene expression correlates with cellular protection and also highlight the potential utility of the inducible $H2K^b$ promoter in the setting of a cytokine-mediated inflammatory response.

DISCUSSION

Expression of human complement inhibitor hCD59 was established in transgenic mice and in a transgenic pig utilizing the murine MHC class I gene as a genomic expression cassette. The proteins encoded by the MHC class I genes from human (HLA), mouse (MHC), and swine (SLA) are expressed in most somatic cell types including the vascular endothelium (22, 24, 26). Therefore, a MHC class I promoter should direct high-level transgene expression in the endothelial cells of vascularized organs. The additional advantage to this genomic expression strategy is that the class I promoter has the capacity to upregulate hCD59 expression in response to the inflammatory cytokines IFN-γ and TNF-α (22, 24, 25).

We have approached the problem of complement-mediated hyperacute rejection during pig-to-primate xenotransplantation by engineering the xenogeneic donor tissue with human complement inhibitor hCD59. The analyses of hCD59 in *H2K^b*–hCD59 transgenic mice and transgenic pig 153-2 demonstrated that the *H2K^b*–hCD59 genomic construct regulated the expression of hCD59 in the context of a transgenic genome. Cell surface expression of hCD59 was detected in a variety of cells and tissues, including the vascular endothelium. The assays used to determine the protective effects of hCD59 expressed on the transgenic cells were performed with human whole serum, which contains serum complement components, as well as high-titer natural antibodies (W.L.F. and S.A.R., unpublished data). In addition, anti-porcine lymphocyte antiserum was used to enhance the activation of the classical complement pathway on the surface of the target cell. Our data demonstrated that the level of hCD59 expressed on the cell surface protected the xenogeneic cell even in the presence of additional complement-activating antibodies.

The utility of blocking complement as a method to prevent hyperacute rejection in pig-to-primate xenotransplantation was demonstrated by using cobra venom factor (CVF) and recombinant soluble complement receptor type 1 (sCR1) (refs. 27 and 28, respectively). A significant delay of complement-mediated hyperacute rejection in pig-to-primate heterotopic cardiac xenotransplantation was observed with the administration of CVF for two consecutive days before transplantation (27) or with a single intravenous bolus of sCR1 before xenograft reperfusion (28). The advantage of developing a transgenic donor animal expressing a human complement inhibitor is to provide the donor tissue with an endogenously expressed membrane-bound inhibitor and therefore does not rely on repeated administration of pharmacological agents.

The successful engineering of transgenic swine expressing a human complement inhibitor, and the demonstration that cells from these animals were significantly protected from human complement attack, suggests that this strategy may represent a useful component of an overall approach to discordant xenotransplantation. This transgenic approach will hopefully make porcine-to-primate transplantation models feasible that will allow the cellular aspects of discordant xenograft rejection to be evaluated. In addition, the production of porcine organs resistant to hyperacute rejection may open therapeutic windows for organ transplantation into humans, particularly when this technology is coupled with advances in cellular immunosuppressive regimens.

We thank Dr. Leonard Bell of Alexion for providing an intellectually stimulating environment and for insightful comments on the work. We would also like to thank Dr. Frank Gwazdauskas, Ed Guilmette, Stella Bianco-Caron, Stephanie DeCesare, and Adeline Tucker for excellent technical assistance.

1. Cooper, D. K. C. (1993) *Xeno* **1**, 25–26.
2. Sommerville, C. A. & D'Apice, A. J. F. (1993) *Kidney Int.* **44**, Suppl. 42, S112–S121.
3. Dalmasso, A. P., Vercellotti, G. M., Fischel, R. J., Bolman, R. M., Bach, F. H. & Platt, J. L. (1992) *Am. J. Pathol.* **140**, 1157–1168.
4. Auchincloss, H., Jr. (1990) *Transplant. Rev.* **4**, 14–27.
5. Platt, J. L., Vercellotti, G. M., Dalmasso, A. P., Mattas, A. J., Bolman, R. M., Najarian, J. S. & Bach, F. H. (1990) *Immunol. Today* **11**, 450–456.
6. Platt, J. L., Lindman, B. J., Chen, H., Spitalnik, S. L. & Bach, F. H. (1990) *Transplantation* **50**, 817–822.
7. Sandrin, M. S., Vaughan, H. A., Dabkowski, P. L. & McKenzie, I. F. C. (1993) *Proc. Natl. Acad. USA* **90**, 11391–11395.
8. Larsen, R. D., Rivera-Marrero, C. A., Ernst, L. K., Cumming, R. D. & Lowe, J. B. (1990) *J. Biol. Chem.* **263**, 7055–7061.
9. Lachmann, P. J. (1991) *Immunol. Today* **12**, 312–315.
10. Rollins, S. A., Zhao, J., Ninomiya, H. & Sims, P. J. (1991) *J. Immunol.* **146**, 2345–2351.
11. Dalmasso, A. P., Vercellotti, G. M., Platt, J. L. & Bach, F. H. (1991) *Transplantation* **52**, 530–533.
12. Walsh, L. A., Tone, M. & Waldmann, H. (1991) *Eur. J. Immunol.* **21**, 847–850.
13. Wing, M. G., Zajicek, J., Seilly, D. J., Compston, D. A. S. & Lachmann, P. J. (1992) *Immunology* **76**, 140–145.
14. Zhao, J., Rollins, S. A., Maher, S. E., Bothwell, A. L. M. & Sims, P. J. (1991) *J. Biol. Chem.* **266**, 13418–13422.
15. Kennedy, S. P., Rollins, S. A., Burton, W. V., Sims, P. J., Bothwell, A. L. M., Squintro, S. P. & Zavoico, G. B. (1994) *Transplantation* **57**, 1494–1501.
16. Weiss, E. H., Golden, L., Zakut, R., Mellor, A., Fahrner, K., Kvist, S. & Flavell, R. A. (1983) *EMBO J.* **2**, 453–462.
17. Hogan, B., Costantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab. Press, Plainview, NY).
18. Church, G. H. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1991–1995.
19. Velander, W. H., Johnson, J. L., Page, R. L., Russell, C. G., Subramanian, A., Wilkens, T. D., Gwazdauskas, F. C., Pittius, C. & Drohan, W. N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 12003–12007.
20. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. & Strober, W. (1992) *Current Protocols in Immunology* (Wiley, New York), pp. 7.1.1–7.1.2; 5.8.1–5.8.2.
21. Madri, J. A., Dreyer, B., Pitlick, F. A. & Furthmayr, H. (1980) *Lab. Invest.* **43**, 303–315.
22. Johnson, D. R. & Pober, J. S. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5183–5187.
23. Kimura, A., Israel, A., Le Bail, O. & Kourilsky, P. (1986) *Cell* **44**, 261–272.
24. Momberg, F., Koch, N., Moller, P., Moldenhauer, G. & Hammerling, G. J. (1986) *Eur. J. Immunol.* **16**, 551–557.
25. Blanar, M. A., Baldwin, S. A., Flavell, R. A. & Sharp, P. A. (1989) *EMBO J.* **8**, 1139–1144.
26. Singer, D. S., Ehrlich, R., Satz, L., Frels, W., Bluestone, J., Hodes, R. & Rudikoff, S. (1987) *Vet. Immunol. Immunopathol.* **1**, 211–221.
27. Leventhal, J. R., Dalmasso, A. P., Cromwell, J. W., Platt, J. L., Manivel, C. J., Bolman, R. M., III, & Matas, A. J. (1993) *Transplantation* **55**, 857–866.
28. Pruitt, S. K., Kirk, A. D., Bollinger, R. R., Marsh, H. C., Jr., Collins, B. H., Levin, J. L., Mault, J. R., Heinle, J. S., Ibrahim, S., Rudolph, A. R., Baldwin, W. M., III, & Sanfilippo, F. (1994) *Transplantation* **57**, 363–370.

NOVEMBER 8, 1994
VOLUME 91
NUMBER 23

Proceedings OF THE National Academy of Sciences

OF THE UNITED STATES OF AMERICA

EXHIBIT E

A Moloney MLV-Rat Somatotropin Fusion Gene Produces Biologically Active Somatotropin in a Transgenic Pig

Karl M. Ebert, Malcolm J. Low, Eric W. Overstrom, Frances C. Buonomo, Clifton A. Baile, Thomas M. Roberts, Alice Lee, Gail Mandel, and Richard H. Goodman

Department of Anatomy and Cellular Biology (K.M.E., E.W.O.)
Tufts University School of Veterinary Medicine
Grafton, Massachusetts 01536

Divisions of Endocrinology (M.J.L.)
and Molecular Medicine (M.J.L., G.M., R.H.G.)
New England Medical Center Hospital
Boston, Massachusetts 02111

Monsanto Company (F.C.B., C.A.B.)
St. Louis, Missouri 63196

Dana-Farber Cancer Center (T.M.R., A.L.)
Boston, Massachusetts 02115

Expression of a Moloney murine leukemia virus (MLV) rat somatotropin fusion gene was examined in a transgenic pig. The fusion gene was integrated in a single site within the genome in a tandem array with approximately eight copies per cell. The integrated MLV-rat somatotropin fusion gene produced high levels of circulating rat somatotropin and resulted in an elevation in the circulating levels of insulin-like growth factor I. Although there was no increase in the rate of growth of the transgenic animal during the rapid growth phase, several phenotypic changes were evident. Skeletal growth was markedly increased and fat deposition was reduced throughout the animal. Blood glucose levels were elevated without ketosis. Northern blot analyses of rat somatotropin RNA revealed that expression of the fusion gene was highest in the spleen, lung, intestine, lymph nodes, and bone marrow. These results show that the MLV promoter can be used to express high levels of biologically active rat somatotropin in transgenic swine. (Molecular Endocrinology 2: 277–283, 1988)

INTRODUCTION

The transgenic mouse model has provided fundamental information on gene expression and regulation (1). Although transgenic mice are convenient for laboratory studies, an important application of genetic manipulation in the future will be to develop the scientific and economic potential of transgenic livestock. In addition to the obvious economic benefits, transgenic domestic animals might provide useful models for studying hormonal abnormalities. Studies with transgenic mice have shown that dramatic phenotypic changes can be produced by introducing genes coding for growth-promoting proteins (2, 3). By analogy, expression of growth-promoting proteins in transgenic domestic animals may allow an improvement in growth characteristics after a single generation rather than the multiple generations required by standard livestock breeding practices. Additionally, these improved growth characteristics may not require the use of feed additives or other medications. Experience introducing foreign genes into domestic animals has been limited, however. The only published report concerning the introduction of foreign genes into any domestic species used the mouse metallothionein promoter linked to the human somatotropin gene (4). Transgenic pigs carrying this fusion gene had elevated levels of circulating human somatotropin. However, unlike the transgenic mice that expressed the same gene (2), the pigs did not show a significant increase in growth. It is possible that the human somatotropin produced by the transgenic pigs was biologically inactive. Alternatively, the mouse metallothionein promoter may not be optimal for expression of foreign genes in transgenic livestock. Therefore, we sought to determine whether a viral promoter-enhancer would be functional and whether a rat somatotropin gene product would be biologically active in transgenic pigs. The Moloney murine leukemia virus (MLV) promoter was

0888-8809/88/0277-0283$02.00/0
Molecular Endocrinology

selected because of its efficiency in gene transfer experiments in a wide variety of cultured cell lines. Additionally, the MLV promoter was predicted to be constitutively active *in vivo*, obviating the need to administer exogenous chemical inducers to the genetically altered animals. Using an MLV-rat somatotropin fusion gene, we produced one transgenic pig which expressed high levels of rat somatotropin. This animal was monitored for phenotypic effects including growth rate, fat production, skeletal growth, and circulating levels of insulin-like growth factor-I (IGF-I). RNA was purified from tissues and used in Northern blot assays to determine the range of expression of the MLV promoter.

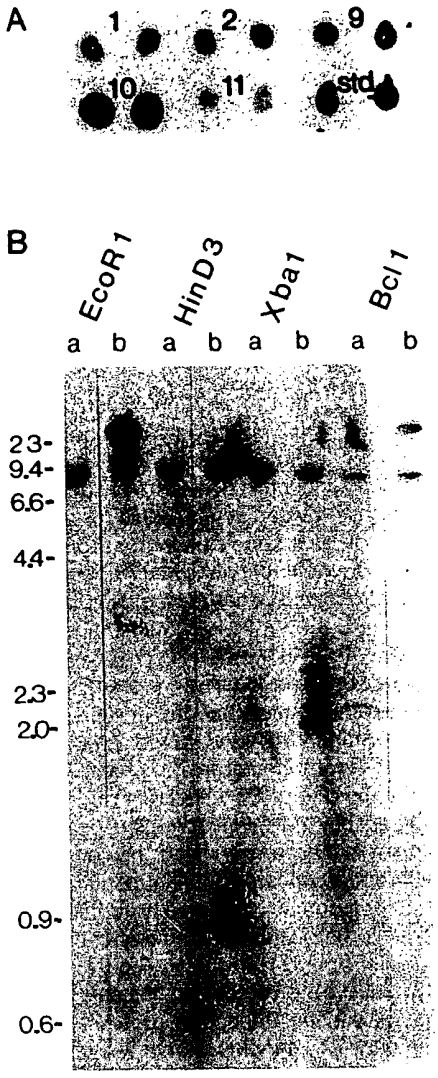


Fig. 1. Identification of Transgenic Swine by Dot Blot Hybridization and Southern Blot Analysis of Genomic DNA

A, Dot-blot from litter 308. Pig 10 contains eight copies per genome of the MLV-rat somatotropin fusion gene. Pigs 1, 2, 9, and 11 show only background hybridization to endogenous somatotropin genes; Std, two copy standard. B, Southern blot. Lane a, Pig 308-11; lane b, pig 308-10; position of *Hin*dIII-digested λDNA and Dde I-digested pUC 12 markers in kb pairs are indicated in the margin.

RESULTS

A total of 100 one-cell and 70 two-cell porcine embryos was collected from 12 Yorkshire gilts. Approximately 2 pl of DNA solution (1.75 ng/μl) containing the Moloney MLV-rat GH (rGH) fusion gene were injected into the male pronucleus of one-cell embryos or into the nucleus of a single blastomere of two-cell embryos.

Of the 15 piglets born, one (no. 308-10) from a litter of four was positive for the MLV-rat somatotropin fusion gene. Dot blot analysis indicated that this pig contained approximately eight copies of the foreign gene (Fig. 1A). Southern blot analysis using DNA extracted from leukocytes of the transgenic pig showed that the endogenous porcine somatotropin gene is represented by an approximately 9-kilobase (kb) fragment in DNA digested with the restriction enzymes *Eco*RI, *Hin*dIII, *Xba*I, and *Bcl*I (Fig. 1B). Electrophoresis of the DNA fragments for a longer period of time indicated that the high molecular weight fragments obtained after restriction endonuclease cleavage were unique for each enzyme. Because the microinjected DNA fragment contains no internal *Eco*RI or *Bcl*I restriction sites, the additional high molecular weight band in the *Eco*RI and *Bcl*I digests indicated a single integration site into the genome of the transgenic pig. *Hin*dIII digestion produced multiple copies of the intact 850 base pair (bp) rat somatotropin cDNA. Digestion with the restriction enzyme *Xba*I resulted in an intensely hybridizing 1900-bp band and a lighter band of 2450 bp which probably represents a junctional fragment. These findings suggest that multiple copies of the MLV-rat somatotropin gene had integrated in a tandem array at a single site within the genome.

Rat somatotropin was measured in serum samples by RIA to determine whether the integrated MLV-rat somatotropin fusion gene was transcribed and translated. The concentration of immunoreactive rat somatotropin in the transgenic pig was 510, 1300, and 500 ng/ml at 2.5, 3.5, and 5.5 months of age, respectively (Table 1). These values were approximately 50-fold higher than in control animals of comparable age. When the pig was killed at 9 months of age its rat somatotropin level was 660 ng/ml.

Despite the high circulating levels of rat somatotropin in the transgenic pig, we could only detect small quantities of the hormone in tissues by RIA. Consequently, we extracted mRNA from many of the tissues and performed Northern blot analyses. These studies showed the highest concentration of rGH mRNA in

Table 1. Rat somatotropin (rGH) and IGF-I levels in plasma from the transgenic pig (308-10) and 31 control pigs (including samples from three negative littermates) averaged over 2.5 to 9.0 months of age

	rGH (ng/ml)	IGF-1 (ng/ml)
Control	15.7 ± 3.39	55.8 ± 7.86
Transgenic	742.5 ± 164.03	379.5 ± 24.78

Levels are the mean ± SE.

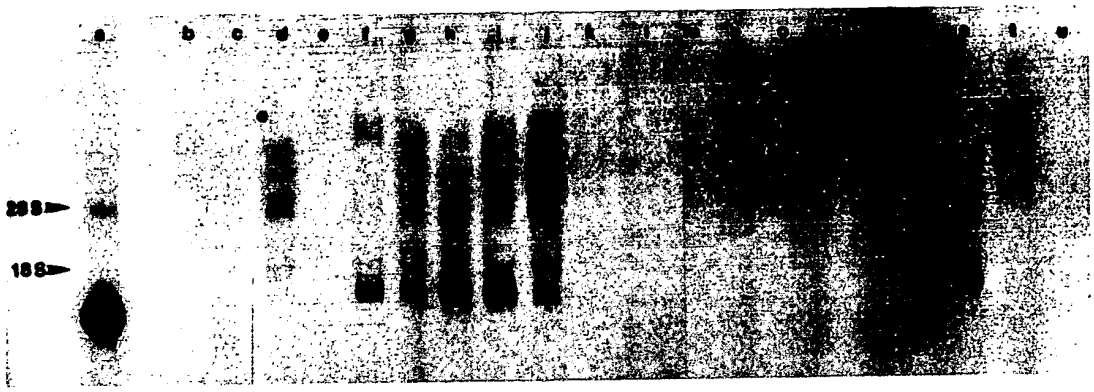


Fig. 2. Northern Blot Analysis of RNA from Pig 308-10

Ten-microgram aliquots of total RNA from each tissue were electrophoresed on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with a radiolabeled rGH cDNA probe. The positions of the 28 S and 18 S ribosomal band are indicated. The predominant hybridizing band in anterior pituitary (lane a) has an estimated size of 1200 bases, consistent in size with the expected polyadenylated porcine GH mRNA. Several other tissues from the transgenic pig have a major hybridizing species of 1600 bases representing the rGH transcript from the MLV promoter. Aside from pituitary, no GH signal was seen in any tissue from control pigs (Low, M. J., unpublished). The lanes are: a, anterior pituitary; b, seminal vesicle; c, testis; d, liver; e, thymus; f, lymph node; g, bone marrow; h, spleen; i, lung; j, kidney; k, adrenal; l, pancreas; m, aorta; n, cerebral cortex; o, tendon; p, tongue; q, stomach; r, colon; s, jejunum; t, skin; u, skeletal muscle.

spleen, lung, colon, and jejunum (Fig. 2). Lymph node, bone marrow, and kidney also contained the fusion gene transcripts, but to a lesser extent. The hybridizing band in the pituitary sample represents the endogenous 1200 nucleotide porcine GH mRNA. Although we were not able to directly compare the levels of pituitary GH mRNA in this transgenic pig to that in controls, subsequent studies have indicated that endogenous GH mRNA is decreased approximately 10-fold in transgenic swine expressing an MLV-porcine somatotropin fusion gene (Ebert, K. M., unpublished). In addition, there was a 10-fold decrease in the number of pituitary cells containing immunoreactive somatotropin in the transgenic swine expressing an MLV-porcine somatotropin gene indicating a negative feedback effect of the ectopic somatotropin on endogenous somatotropin biosynthesis. A similar decrease in endogenous pituitary somatotropin gene expression in transgenic mice was reported previously (5). The predominant band in other tissues has a mol wt of 1600 nucleotides due to the 400 bases of transcribed MLV sequences between the LTR and rat somatotropin cDNA.

IGF-I, a somatotropin-dependent circulating growth factor, was measured in the transgenic pig as an indicator of the biological activity of the rat somatotropin. Serum levels of IGF-I in pig no. 308-10 were 323, 373, 459, and 363 ng/ml at 2.5, 3.5, 5.5, and 9 months, respectively. These values were approximately 7 times that of age-matched controls (Table 1).

Weights were determined every 2 weeks on pig no. 308–10 and the three negative littermates. Despite the high levels of circulating IGF-I, the weight of pig no. 308-10 was only slightly higher than that of the three normal littermates during the rapid growth phase. The average weights of the three normal littermates at 2.5, 3.5, and 5.5 months of age were 27.9, 45.3, and 94.2 kg, respectively. The corresponding weights of pig no. 308-10 were 31.6, 48.2, and 104.5 kg, respectively. However, by 9 months of age no. 308-10 was 26% heavier (200 kg *vs.* 159 kg) than the normal male littermate.

Clinical laboratory analysis revealed glycosuria (1000 mg/dl) and a serum glucose level (357 mg/dl) that was approximately 3 times the normal serum level (6). This elevated glucose level was consistent throughout the life span of the animal. Serum electrolytes and liver function studies were normal (data not shown). Testosterone values in the transgenic pig were 6.90 and 2.29 ng/ml at 6.5 and 9.0 months of age, respectively, and were considered normal.

Although the weight of pig no. 308-10 during the rapid growth phase was only slightly increased, several phenotypic changes were evident. Radiographic analyses of the fore and hindlimbs showed increased linear bone growth, increased transverse diameter, and thinned cortices in the metacarpal bones compared to the three control littermates (Fig. 3). Bone age, assessed by epiphysial closure, was not altered. Additionally, the depth of backfat at age 5 months, determined by ultrasonography, was dramatically reduced in the transgenic animal, as compared to the normal male littermate (data not shown). Postmortem examination confirmed the absence of fat (Fig. 4) and revealed extensive joint pathology characteristic of osteochondritis dissecans (7; Fig. 5). Examination of the testes showed tubular atrophy and a marked reduction of spermatogenesis.

DISCUSSION

A transgenic pig containing an MLV-rat somatotropin fusion gene was obtained by microinjecting the gene

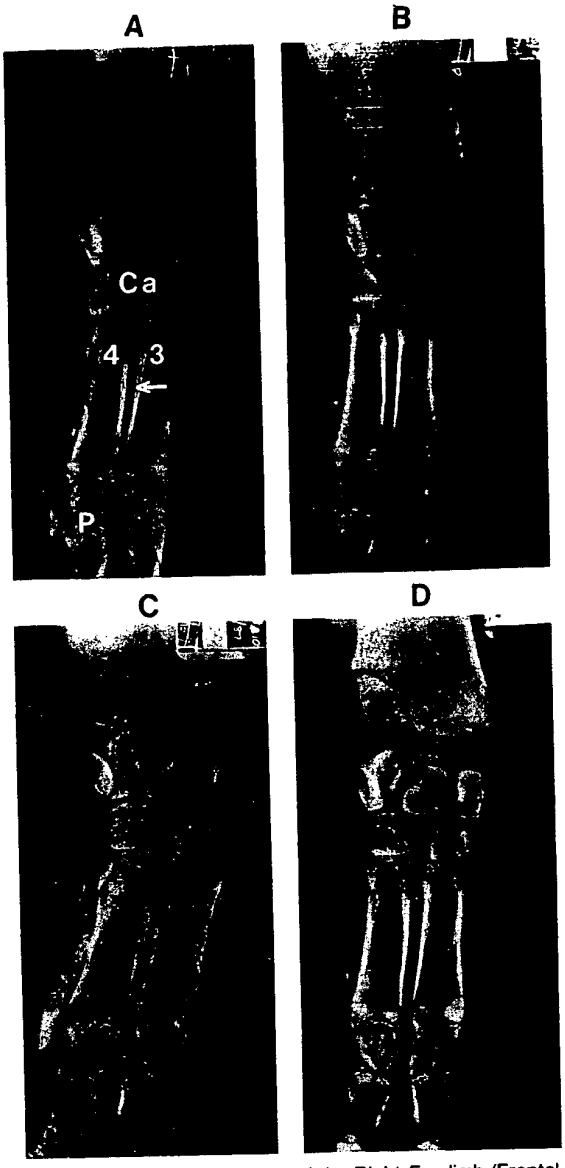


Fig. 3. Radiographic Analyses of the Right Forelimb (Frontal View) of Litter 308

A, 308-1; B, 308-2; C, 308-10; D, 308-11. The x-ray of the transgenic male (308-10) shows an increase in the length and width of the metacarpal bones (3 and 4) and a decrease in the thickness of the cortex (*arrow*). Carpals (Ca) and phalanges (P) are also noticeably larger than in the littermates. Hindlimb x-rays showed essentially the same changes.

into the male pronucleus of a porcine zygote. The gene appeared to be integrated into a unique site within the genome, consistent with observations in transgenic mice (1). The MLV promoter is transcriptionally active in pigs, as shown by the high levels of circulating rat somatotropin. The human somatotropin gene under the control of the mouse metallothionein promoter, has also been shown to be expressed in transgenic pigs (8). Unlike the metallothionein fusion genes however (9), the MLV-fusion gene examined in our studies was expressed primarily in hematopoietic tissues and a

Fig. 4. Cross-Section through the Midlumbar Region of the Transgenic Pig (308-10), Top; and the Nontransgenic Negative Male Littermate (308-11), Bottom

Note the dramatic decrease in the amount of backfat (*arrows*) in the transgenic pig.

subset of nonhematopoietic tissues including kidney, lung, and intestine. This pattern of expression overlaps with the natural tropism of the intact retrovirus in mice. Little expression was detected in liver, the major site of metallothionein-fusion gene expression.

Elevated levels of IGF-I were detected in the transgenic pig indicating that the rat somatotropin was biologically active. This result appears to conflict with an earlier report suggesting that exogenous rat somatotropin given by injection is not biologically active in hypophysectomized swine (10). It is possible that this difference is due to the extremely high and sustained levels of somatotropin produced in the transgenic pig. Previous studies have also shown that injections of human, rat, and even porcine somatotropin only minimally affect weight gain during the rapid growth phase of swine (11, 12). It was not surprising, therefore, that the transgenic pig showed a normal rate of growth during its rapid growth phase (2–6 months). The observation that the transgenic pig continued to gain weight at a continuous rate from 2–9 months of age while the normal littermates decreased their rate of growth after 6 months suggests that continuously elevated levels of rat somatotropin may have the potential to augment the growth of swine during a later phase of development.

The dramatic effects of the rat somatotropin levels on skeletal growth and backfat indicate that major

Fig. 5. The Left Humeral Condyles from the Transgenic Pig 308-10
There were several loose, white cartilage bodies on the glenoid surface (*arrow*), a condition that is indicative of osteochondritis dissecans.

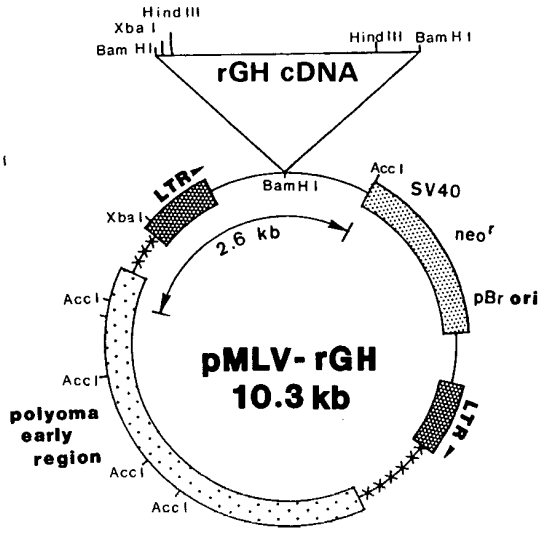


Fig. 6. Structure of the MLV-Rat Somatotropin (MLV-rGH) Fusion Gene
An 850 bp fragment of the rat somatotropin cDNA containing the entire protein coding sequence was ligated into the unique *Bam*HI site of the shuttle vector pDOL downstream of the MLV 5′-LTR. Restriction endonuclease sites used for cloning and Southern blot analysis are indicated. A 2.6-kb *Acc*I fragment indicated by the *double-headed arrow* was used for microinjection into porcine zygotes.

phenotypic changes can occur in transgenic livestock. The reduction in carcass fat within the transgenic pig is similar to the fat depletion seen in pigs injected daily with porcine somatotropin (13) and is probably due to a direct action of somatotropin itself. It is possible, however, that the decrease in fat deposition is related in part to the coincidental diabetes mellitus. The increase in long bone growth and the joint pathology may be due to either the elevated levels of somatotropin or IGF-I. Similar but less dramatic osteochondritis has been detected in several nontransgenic animals from our herd at comparable age and may indicate a normal condition which is enhanced by elevated GH (7). Also, identical phenotypic changes (*i.e.* decreased fat deposition and joint pathology) have been noted in other transgenic pigs expressing an MLV-porcine somatotropin fusion gene (Ebert, K. M., unpublished).

Chemical profiles of the transgenic animal also show typical endocrine effects of high levels of somatotropin. Elevated blood glucose is a common feature of acromegaly in humans and is thought to be due to insulin resistance.

Our studies indicate that major phenotypic changes can be produced in transgenic livestock through expression of microinjected fusion genes. Overproduction of rGH in the transgenic pig led to several desirable, and some clearly undesirable new traits. The full potential of this technology will be realized only when it becomes possible to precisely regulate the expression of the microinjected genes.

MATERIALS AND METHODS

Construction of the MLV-rGH Fusion Gene

An 850 bp *Hin*dIII restriction fragment of the rat somatotropin cDNA containing the entire protein coding sequence (14) was adapted for ligation into the unique *Bam*HI site of the shuttle vector pDOL (15) downstream of the MLV 5′-LTR (Fig 6).

*Bam*HI cohesive ends were obtained by initially cloning a 1470 bp *Cla*I-*Acc*I fragment containing the rat somatotropin cDNA into the plasmid pUC 12, and then isolating the rat somatotropin sequence by *Bam*HI digestion. In the resulting 1200-bp *Bam*HI fragment, the rat somatotropin cDNA is flanked by a short polylinker sequence at its 5′-end and by 345 bp of pBR322 at the 3′-end. Restriction endonuclease sites used for cloning and Southern blot analysis are indicated in Fig. 6. A 2.6-kb *Acc*I fragment was used for microinjection into the porcine zygotes.

Production of Transgenic Pigs

Twelve Yorkshire gilts were used for collection of one-cell and two-cell porcine embryos. The gilts were synchronized and superovulated with a modified feeding regime of Webel (16). The animals were fed a synthetic orally active progestin, Altrenogest (Regu-Mate, Hoechst, West Germany), at a concentration of 15 mg in 1.8 kg feed/day for 9 days beginning on days 12–16 of the estrous cycle. Ovulation occurred approximately 5 days after the last feeding of progestin. Gilts were superovulated by injecting sc 2000 U PMSG (Sigma, St. Louis, MO) 24 h after the last feeding of Altrenogest, followed 78 h later with an im injection of 1000 U human chorionic gonadotropin (hCG, Sigma). This regime produced an average of 28 ovulations per animal. Gilts were bred by artificial insemination with sperm from proven boars at 24, 36, and 48 h after injection of hCG.

Embryos were collected from gilts under aseptic conditions by cannulating the ampulla region of the oviduct and flushing the oviduct with 10–15 ml Kreb's Ringer-Bicarbonate solution. The embryos were initially maintained at 30 C, then were transferred to Ham's F12 medium containing 10% fetal calf serum and were incubated at 38 C in 5% CO_2. Before injection, the zygotes or two-cell embryos were centrifuged at 13,000 × *g* in an Eppendorf centrifuge to visualize the pronuclei or nuclei, respectively. The male pronucleus or one nucleus of the two-cell embryo was injected with approximately 2 pl plasmid solution using interference contrast microscopy. The injection was done in a droplet of modified BMOC-2 medium containing HEPES salts (17). The injected zygotes were stored in an incubator at 38 C until transfer to a recipient female.

DNA Ear Blot Hybridization

DNA was extracted from an ear punch (18), quantified using the Hoechst dye method (19), and 1.8 μg were dotted in duplicate onto nitrocellulose. The dot blot was probed with a cDNA encoding the porcine somatotropin coding sequences (Mandel, G., unpublished), radiolabeled to a specific activity of 10^9 cpm/μg by random hexanucleotide priming (20). This cDNA insert, confirmed by nucleotide sequencing (21), hybridizes equally well to rat and porcine somatotropin genomic sequences. Two copies of the integrated gene were calculated to be equivalent to 0.5 pg of an 850-bp rat somatotropin cDNA restriction fragment. Copy number in the pigs was determined by scintillation counting of the DNA dot pairs and comparison of counts obtained from genomic DNA to the standards. A normal pig was assumed to contain two alleles of a single copy somatotropin gene.

Southern Blot Analysis of the MLV-rGH Fusion Gene

High molecular weight DNA was extracted from peripheral leukocytes (22) and 10-μg samples were digested to completion with the indicated restriction endonucleases. The samples were electrophoresed on a 0.7% agarose gel for 15 h at 50 V, transferred by electroblotting to a Zetabind membrane, and prehybridized in 6× SSC, 1% sodium dodecyl sulfate, (SDS), and 50 μg heparin/ml at 65 C for 1 h.

Hybridization was performed in 6× SSC, 1% SDS, and 500 μg heparin/ml using 1.5×10^6 cpm porcine somatotropin cDNA probe/ml. The filter was washed in 0.2× SSC, 0.2% SDS at 65 C, and autoradiographed for 24 h with an intensifying screen.

Northern Blot Analysis of the rGH mRNA

Total cellular RNA was prepared from frozen tissues by extraction in 5 M guanidine thiocyanate, 20% β-mercaptoethanol (vol/vol), 10 mM EDTA, 50 mM Tris-HCl, pH 7,5, followed by precipitation in 4 M LiCl (23). The quality of the RNA was checked by ethidium bromide staining of the 28 S and 18 S ribosomal bands on a native agarose gel. Ten micrograms of total RNA were electrophoresed on a 1% agarose-formaldehyde gel and transferred by electroblotting to a Zetabind (American Bioanalytical, Natick, MA) membrane. Prehybridization and hybridization conditions were performed as previously described (24). A rat somatotropin cDNA probe was radiolabeled as described above. The molecular weight of hybridizing bands was estimated from the positions of the 28 S and 18 S ribosomal bands and an RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD).

RIAs

Circulating rat somatotropin was measured by RIA according to Glenn (25). The cross-reactivity of the antibody with porcine somatotropin was 60%. Serum samples were taken at 1-month intervals beginning at 2 months of age until the animal was killed. As only one blood sample was taken at each time, the fluctuations in endogenous somatotropin were not considered and blood samples from 31 control pigs including samples from three negative littermates were averaged over 2.5 to 5.5 months of age for a control value.

IGF-I was measured in the same samples using a heterologous human IGF-I RIA as outlined by Buonomo *et al* (26).

Acknowledgments

The authors would like to acknowledge A. Schmidt, T. Smith, S. Hagopian, R. Felix and C. Banks for their excellent technical assistance. We would also like to thank Dr. J. Erickson, G. Miller and K. Baldwin for their help with the synchronization, superovulation, and artifical insemination of the swine and animal husbandry that is so critical to a successful program with research using farm animals, and Drs. G. Meuller and M. S. A. Kumar for the Northern Blot and testosterone measurements, respectively. The authors would also like to acknowledge the Veterinary staff at the Tufts University School of Veterinary Medicine, especially Drs. H. Ernst and S. Schelling, for their assistance in the medical aspects of this study. Acknowledgment is also extended to J. Ebert for assistance with the preparation of this manuscript. The authors have complied with the appropriate experimental procedures for the care and use of animals as stated by the *American Journal of Physiology*.

Received November 9, 1987. Accepted December 16, 1987.

Address requests for reprints to: Dr. Karl M. Ebert, Department of Anatomy and Cell Biology, Tufts University School of Veterinary Medicine, Grafton, Massachusetts 01536.

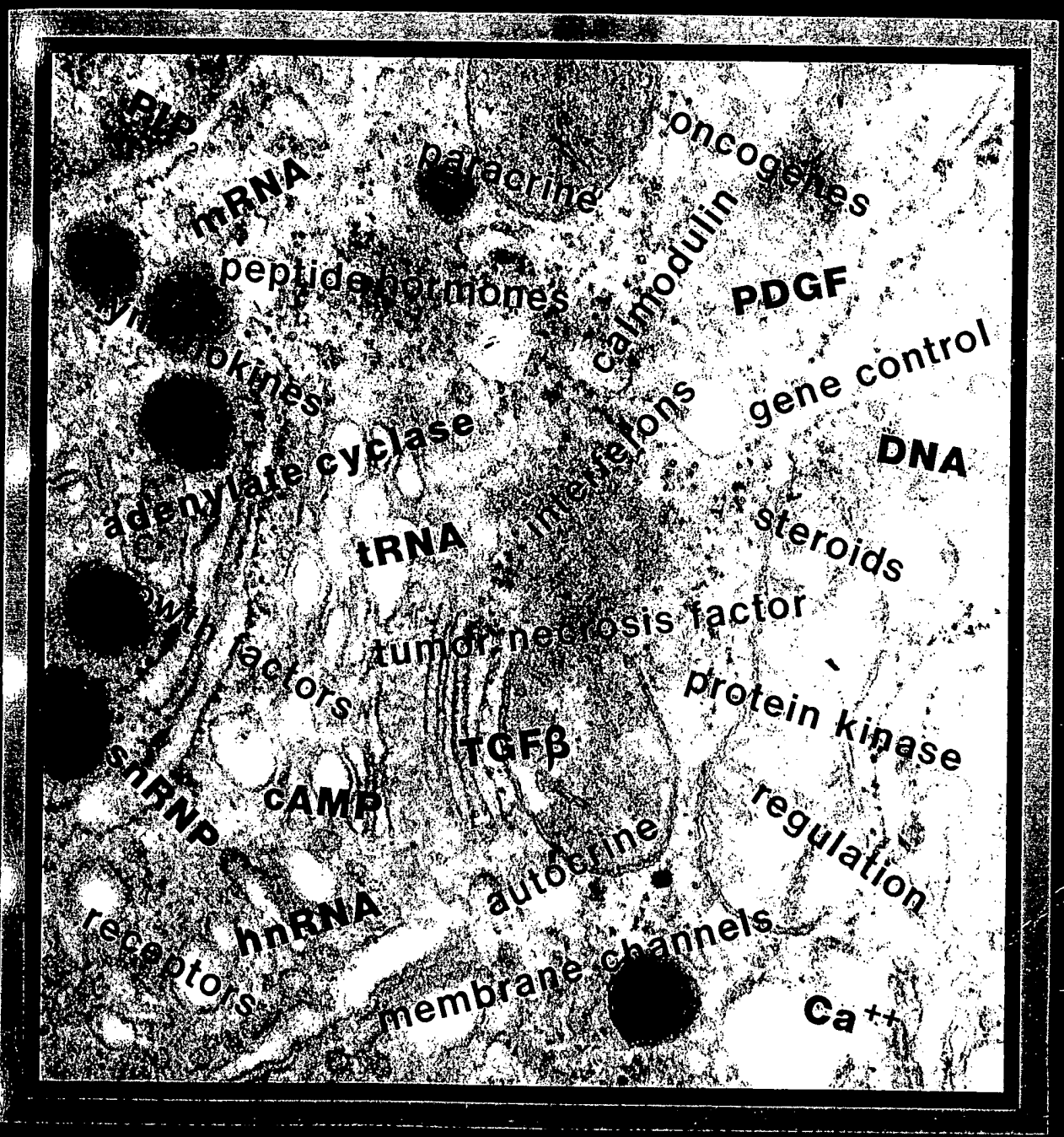
This work was funded by a grant from the Monsanto Company.

REFERENCES

1. Palmiter RD, Brinster RL 1986 Germ-line transformation of mice. Annu Rev Genet 20:465–499
2. Palmiter RD, Brinster RL, Hammer RE, Trumbauer ME,

MOLECULAR ENDOCRINOLOGY

Volume 2, Number 3 March 1988



ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY

US 099646780EP1

Creation date: 09-11-2003
Indexing Officer: RNEFTALIEM - RAHEL NEFTALIEM
Team: OIPEBackFileIndexing
Dossier: 09964678

Legal Date: 10-18-2002

No.	Doccode	Number of pages
1	SRNT	1

Total number of pages: 1

Remarks:

Order of re-scan issued on .....